STUDIES ON DEVELOPING LIMBS IN CHICK AND MOUSE EMBRYOS

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DISCUSSION

SUMMARY

1. Differentiation of epiphyseal cartilage in developing hind limb-buds of chick and mouse has been studied, both by light and electron microscopy. Similar studies have been carried out on cartilage of chick differentiated in tissue culture. Results were also obtained by autoradiography using proline-H^3 in tissue culture, cytochemistry for glycogen and RNA, and immunology. Effects of hydrocortisone on developing limbs of the chick were also studied.

2. The results indicate that during differentiation of chondrogenic cells the endoplasmic reticulum and the Golgi apparatus become very well developed. There is also a decrease in the nucleo-cytoplasmic ratio, and the nucleus no longer occupies the centre of the cell. The general electron density of the cells increases and there is a change in the outline of the cell from smooth to scalloped. Some cytosomes (i.e. vacuolar bodies which may contain lipids, vesicles or other unidentified structures) are formed.

3. The endoplasmic reticulum in chondroblasts as well as in chondrocytes shows three types of profile - rough cisternal, both elongated and saccular (up to c.5 μ across) and smooth vesicular - all filled with moderately electron-dense amorphous material. The cisternae have been seen to be directly continuous with the outer nuclear membrane, Golgi apparatus and plasmalemma, indicating that the various components of the membrane system are to some extent interconvertible. Feed-back control of nuclear activity by the contents of the endoplasmic reticulum is suggested.

4. The juxtanuclear Golgi apparatus of cartilage cells shows three types of profile - lamellar, vesicular and large vacuolar (up to 1.2 μ across). The lamellae and the vesicles contain moderately electron-dense material, but the vacuoles are usually electron-translucent in early chondroblasts and contain a chondrogen granule, in late chondroblasts and chondrocytes. The substructure of the chondrogen granule shows smaller granules with attached fibrils, which resemble similar elements of the extracellular phase.

5. The synthesis of glycogen is a feature of chondrogenesis in the epiphyseal cartilage of the mouse, and the amount of glycogen increases with progressive differentiation. In contrast, only a small amount of glycogen was seen in chick, and it was confined to a few cells of diaphysis.

6. Chondroblasts differentiated in tissue culture, as compared to those differentiated <u>in vivo</u>, have a greater number of cytosomes and often show some intracytoplasmic fibrils. The contents of the cytosomes are also more heterogeneous.

7. In the last stages of differentiation, two types of hypertrophy are found. In the mouse, there is a progressive increase in the general electron density and in glycogen content of the cells, while in the chick, the general electron density reaches a maximum in chondrocytes and decreases during hypertrophy, so that the hypertrophied chondrocytes appear rather electron-translucent; and, their cytoplasmic organelles also undergo degeneration.

8. The amount of the extracellular phase increases with progressive differentiation and is very extensive in fully-formed cartilage. In the

mesenchyme the extracellular phase is hyaline, but in the fully-formed cartilage, it appears under the electron microscope as an amorphous ground substance with fibres and granules. The fibres are usually 15 - 20 mμ thick and some of them show a faint periodicity, of 9 mμ in chick, and 5.5 mμ in mouse. In chick chondrogenesis the fibres appear slightly earlier than the granules. The chemical nature of the fibres and granules is discussed and it is suggested that the fibres are almost certainly collagenous, whereas the granules contain accumulations of protein-polysaccharides.

9. Synthesis and secretion of the contents of the extracellular phase have been investigated, both by autoradiographic and morphological studies, and the findings are discussed with particular reference to the contents of the Golgi vacuoles and endoplasmic reticulum, and to the process of excortication. It is suggested that, in cartilage, the non-collagenous protein, after synthesis in the endoplasmic reticulum, is combined with the polysaccharides in the Golgi apparatus and then secreted; whereas the collagen is directly secreted out of the endoplasmic reticulum.

10. The immunological studies indicate that the saline-soluble fraction of embryonic chick cartilage is very weakly antigenic.

11. Hydrocortisone acetate, when injected into 3 or 4-day-old chick embryos, caused necrosis in limb-bud mesenchyme (primary effect), after a further incubation of 24 hours. The necrotic centres usually appeared in the central and subapical mesenchyme. Similar treatment of 4-day-old embryos with higher doses (7.5 mg/egg) caused, in addition, haemorrhage in limb-buds, micromelia (secondary effects), and a slight retardation in growth. The mechanisms of

phagocytosis and necrosis, due to hydrocortisone treatment, were investigated and are discussed.

12. The distribution of RNA in the chick limb-buds was studied, using methyl green-pyronin staining and RNAse digestion.

INTRODUCTION

These investigations on the limb-buds of higher vertebrates were undertaken to study firstly, the normal development with special reference to cartilage and secondly, the ways in which the normal limb development is affected by extragenetic factors which cause various congenital and other malformations. Cartilage was chosen for this study since it is the main, and a ubiquitous, skeletal tissue of the vertebrate embryo and it is the ontogenic predecessor of most bones which form the skeletal tissue of adults. Of the extragenetic factors hydrocortisone was chosen because of its specific effects on the mesoderm.

Fell (1925), Follis and Berthrong (1949) and Godman and Porter (1960) in their publications include brief reviews of earlier work on cartilage, extending back to the reports on its cellular nature (Schwann, 1847), lipid and glycogen content (Leydig, 1857 and Rouget, 1859, respectively) and general histology of its resorption in avian embryos (Strelzoff, 1873).

Several workers have done electron microscopic studies on fully-differentiated cartilage from various mammalian sources, e.g. epiphyseal cartilages of kitten (Scott and Pease, 1956), rat (Policard and Bôud, 1958; Tousimis and Follis, 1958; Godman and Porter, 1960) and mouse (Takuma, 1960); articular cartilages from mouse (Zelander, 1959; Silberberg, Silberberg, Vogel and Wettstein, 1961; Silberberg, Silberberg and Feir, 1964; see also review by Silberberg, 1968), rat and guinea pig (Zelander, 1959) and rabbit (Davies, Barnett, Cochrane and Palfrey, 1962; Palfrey and Davies, 1966); ear cartilages of rabbit (Sheldon and Robinson, 1958; Sheldon and Kimball, 1962), rat (Anderson, 1964) and

human infant (Goshi, 1966); the induced cartilage in mouse (Anderson, 1967) and cartilage from mandibular condyle of pig (Silva and Hart, 1967). Anderson (1964) also studied the femoral and tracheal hyaline cartilage of rat, and Bonucci (1967) studied the calcifying epiphyseal cartilage from guinea pig and rat. Cartilage from regenerating limbs of Amblystoma has been studied by Hay (1958) and Revel and Hay (1963). Glauert, Fell and Dingle (1969) studied the effect of sucrose in culture medium on the ultrastructure of chick cartilage cells. Matukas, Panner and Orbison (1967) in addition to making some very brief remarks on cartilage cells, studied mainly the extracellular phase of cartilage from tibia of chick. Moreover, studies concerned almost exclusively with the extracellular phase of cartilage have also been done at electron microscopic level by following workers: Randall, Fraser, Jackson, Martin and North (1952, joint surfaces of adult fowl, elephant and man, nasal cartilage of calf and femur of chick embryo), Martin (1954, tibial cartilage of fowl), Robinson and Cameron (1956, 57, articular and epiphyseal cartilage of human infant), Luft (1965, frog xiphoid cartilage), Smith, Peters and Serafini-Fracassini (1967, bovine articular and nasal cartilage), and Pihl, Gustafson and Falkmer (1968, rabbit ear cartilage).

The differentiation of cartilage from mesenchymal tissue at electron microscopic level, however, has been studied only by Hay (1958) and Godman and Porter (1960). Review papers concerning cartilage have been published by Cameron (1963), Jackson (1964), Porter (1964), Sheldon (1964) and Silberberg (1968); Person and Philpot (1969) have reviewed invertebrate cartilage.

Cartilage consists of cartilage cells, the chondrocytes, interspersed in an extensive amount of extracellular phase; cartilage has the most abundant extracellular phase of all mature connective tissues (Godman and Porter, 1960).

The ultrastructure of chondrocytes from various sources differs only moderately. A chondrocyte is usually a more or less rounded or oval cell with small, shallow indentations and has a well-defined plasmalemma. In a number of cases the cells are surrounded by a lacunar space, which is regarded by some workers as a fixation artefact (Robinson and Cameron, 1957; Anderson, 1964). The cytoplasm has a well developed endoplasmic reticulum and a juxtanuclear Golgi apparatus. Various types of cytosomes - multivesicular bodies and vacuolar pools, etc., are also found in most chondrocytes. Some chondrocytes also contain cytofibrils (Meachim and Roy, 1967). In addition a dense felt work of material is reported in chondrocytes from elastic cartilage (Sheldon and Robinson, 1958; Sheldon 1964).

The presence of glycogen in the cytoplasm of cartilage cells has been histochemically demonstrated by several workers (Rouget, 1859; Harris, 1932; Follis and Berthrong, 1949; Montagna, 1949; Anderson, 1964). Montagna (1949) stated that in cartilage cells stored glycogen is "a normal and constant cellular component" and that "in adult mammalian tissues only liver and muscle contain more glycogen than cartilage".

The presence of lipids in cartilage cells has also been long recognised (Leydig, 1857) but detailed histochemical studies of lipid distribution have been done only comparatively recently (Sheehan, 1948; Follis and Berthrong, 1949; Montagna, 1949; Collins, Ghadially and Meachim, 1965). These studies show that lipid droplets of various types - neutral fats, fatty acids and phospholipids, "are a normal and constant cellular component" of cartilage cells (Montagna, 1949).

The structure, chemical composition and biosynthesis of the extracellular contents of cartilage has attracted considerably more attention than the chondrocytes themselves. At the ultrastructural level, the extracellular phase generally consists of a ground substance with granular and fibrous elements. Sheldon and Robinson (1958) and Anderson (1964), working on rabbit and rat ear cartilage respectively, reported an electron dense 'felt-like' elastin, present in the extracellular phase. The ground substance is amorphous, homogeneous and electron translucent but may sometimes contain floccular masses of moderately electron dense material. The frequency and width of granular and fibrous materials, as well as the substructure of the fibres, vary considerably depending on the type of developmental stage of cartilage (see Table I). However, Durning (1958) from observations on frozen-dried material considered the matrix to be composed of "a homogeneous component of moderate density and a series of long sheets (about 500 Å thick) interconnected by several orders of decreasingly thin sheets".

The chemical composition of the extracellular phase has been studied by tissue homogenization and fractionation (Meyer, Davidson, Linker and Hoffman, 1956; Dische, Danilczenko and Zelmnis, 1958; Eastoe, 1961; Szirmai, Tyssonsk and Gardell, 1967), as well as by histo- and cytochemistry (Follis and Berthrong, 1949; Montagna, 1949; Marinozzi, 1960; Revel, 1964; Ghadially, Meachim and Collins, 1965; Williamson and Vaughan, 1967; Matukas et al., 1967).

From tissue homogenization and fractionation work, it is fairly well established that there are three main chemical constituents of the extracellular phase, i.e. collagen, protein-polysaccharides, sometimes referred to as chondromucoproteins, and interstitial fluid (see Eastoe, 1961; Jackson, 1964);

in some cases, e.g.,epiphyseal cartilages of young rabbit and pig, a glycoprotein containing sialic acid (sialoprotein) has also been reported (Castellani, Ferri, Bolognani and Graziano, 1960; see also Williamson and Vaughan, 1967). Collagen, about 40% of the dry weight of cartilage, is present either in an insoluble fibrous form or in a soluble nonfibrous form called tropocollagen. The protein-polysaccharides, up to 50% of the total dry weight of cartilage, consist of a noncollagenous protein and polysaccharides. The polysaccharides are the acid mucopolysaccharides, mainly chondroitin sulfate A and C., minor amounts of some other acid mucopolysaccharides, keratosulphates and hyaluronic acid (Szirmai et al., 1967), and some neutral mucopolysaccharides (Dische et al., 1958) are also reported. The interstitial fluid of the extracellular phase contains free electrolytes, plasma proteins, hormones, amino acids, sugars, peptides, and precursors and degradation products of other compounds (Jackson, 1964).

From histochemical studies it is well known that staining of cartilage matrix with toluidine blue results in metachromasia indicating the presence of sulphated esters of polysaccharides in the tissue. Revel (1964), using colloidal-thorium (thorotrast) staining in the electron microscopic studies on various cartilages, also demonstrated the presence of acid mucopolysaccharides in the matrix. Marinozzi (1960) by using periodic acid-silver technique demonstrated the presence of neutral mucopolysaccharides in the cartilage matrix. The sudanophilic nature of the matrix of human tracheal cartilage, due to the presence of lipids, is pointed out by Montagna (1949). Ghadially et al.(1965) also showed the lipid content of matrix in human articular and some other cartilages, by using fat-soluble oil red O. No cytochemical work has been done on the presence of collagen in the matrix.

The chemical interpretation of the ultrastructure of the matrix is still dubious (Cameron, 1963; Matukas et al., 1967). It is usually considered that the amorphous ground substance represents the protein-polysaccharides, neutral polysaccharide-protein complexes, interstitial fluid and tropocollagen (Godman and Porter, 1960; Sheldon, 1964). The electron dense granules of the matrix were earlier regarded either as mineral deposits (Godman and Porter, 1960) or as a canalicular system in section (Cameron and Robinson, 1958). Revel and Hay (1963) and Luft (1965) suggested them to be of polysaccharide nature: and Matukas et al.(1967) confirmed the presence of acid mucopolysaccharides in the granules of the matrix by cytochemical means. All the fibrous material of the matrix is usually considered to be collagen (Godman and Porter, 1960 among others); however, 64 mμ peridicity, characteristic of native collagen, in case of intact cartilage is observable only in a few instances (Table I).

The history of fibrogenesis from the middle of the 18th century has been reviewed by Jackson (1964) and briefly summarised by Godman and Porter (1960). At the turn of the present century it was generally believed that fibres are extracellular, but there were controversial views concerning the production of fibres: some regarded the fibres to be extensions of cartilage cells (Retterer, 1917), others considered the fibres to develop extracellularly from products of non-cartilage cells, for example, fibrin of blood plasma (Baitsell, 1915; Nageotte and Guyon, 1934), while still others regarded them to be a product of cartilage cells (Fell, 1925). Although the autoradiographic work, involving the synthesis of collagen by chondroblasts (Young, 1962; Revel and Hay, 1963; Cooper and Prockop, 1968), fibroblasts and odontoblasts (Ross and Benditt, 1965; Reith, 1968, among others), has shown that collagen is produced by the connective

tissue cells themselves, the primary site where collagenous protein takes its ultrastructurally visible fibrous form has not yet been identified despite the high resolving power available in the electron microscope (see reviews by Cameron, 1963; Jackson, 1964).

Concerning the biosynthesis and secretion of extracellular materials, the evidence from studies on cartilage (Hay, 1958; Godman and Porter, 1960; Silberberg et al, 1961; Campo and Dziewiatkowski, 1962; Sheldon and Kimball, 1962; Revel and Hay, 1963; Godman and Lane, 1964; Fewer, Threadgold and Sheldon, 1964; Peterson and Leblond, 1964) and other tissues (Caro and Palade, 1964; Ross and Benditt, 1965; Jacob and Jurand, 1965; Perry and Waddington, 1966; Kessel, 1966; Jamieson and Palade, 1967; Beam and Kessel, 1968; Manasek, 1968) has been generally interpreted as follows. The proteins needed for intracellular use are believed to be synthesized by polysomes lying free in the cytoplasm (Karasaki, 1964; Porter, 1964; Fawcett, 1966; Manasek, 1968). The proteins needed for export to the extracellular sites, on the other hand, are considered to be synthesized by polysomes that are attached to the endoplasmic reticulum. The product is transferred from the cisternae of the granular endoplasmic reticulum to the Golgi apparatus. The vacuoles of the Golgi apparatus migrate to the cortical parts of the cell, their membranes fuse with the plasmalemma and eventually form an opening or stoma with the extracellular phase where they secrete their contents by way of merocrine secretion. The addition of the carbohydrate moiety to protein, if it occurs, takes place in the area of Golgi apparatus. The details of various processes which may be possibly used by the cell to elaborate the extracellular substances, specially collagen protein, have recently come under renewed discussion.

Studies on the differentiation of cartilage in tissue culture started with the work of Strangeways and Fell (1926) and Fell (1928). In their work mesenchymal tissue from chick limb-buds was allowed to grow and differentiate into cartilage on a blood plasma-embryo extract clot. Fell and co-workers (from Strangeways Research Laboratory, Cambridge) as well as others have since been using tissue differentiated *in vitro* to study various problems concerning cartilage and bone (Fell, 1933, 40, 53; Caplan, Zwilling and Kaplan, 1968; Zwilling, 1966; Fell and Dingle, 1969, among others). With this approach it has been possible to evaluate, for the first time, the potency of various cells to form different tissues independent of the influences operating *in vivo*; for example, Jacobson and Fell (1941), working on chick mesenchymal tissue grown *in vitro*, showed "that the three types of mesenchymal cells in the mandible - myogenic, chondrogenic, and osteogenic - are independent in origin, distinct in time of appearance, and already determined while being formed". Zwilling (1966), also working on chick mesenchymal tissue, on the other hand, pointed out that in case of limb-buds at stage 22 to 24 the frequency and rate of cartilage formation in tissue culture was independent of whether the mesenchyme was chondrogenic or myogenic in origin (see also Medoff, 1967).

Moscona and Moscona (1952) introduced a new approach to the differentiation of cartilage in a liquid culture medium. These authors dissociated the mesenchymal cells from limb-buds of 4-day-old chick embryos by treatment with trypsin, and allowed these discrete cells to reaggregate in suspension cultures. Their results show that it is possible to get a characteristic histotypically differentiated cartilage from completely dissociated chondrogenic tissue in cultures. Further experiments show that depending on whether the cells had been taken from a limb-bud or the prescleral coat around the eye, the disaggregated prechondrogenic cells

differentiate to express the typical architectural characteristic of either limb or scleral cartilage, respectively, that is, give rise to massive whorl-shaped patterns in the former case, and laminated sheets in the latter (see Jackson, 1965; Weiss, 1965). Lately the improvements in tissue culture media have further added to the usefulness of this technique and its widespread use. Several workers have used tissues grown in vitro to test the effect of various chemicals on development, and on the synthesis of various extracellular materials, etc. (Stefanelli and Zacchei, 1958; Okada, 1960; Medoff, 1967; Reynolds, 1967; Fell and Dingle, 1969; Glauert et al, 1969).

Immunological work on cartilage is very scanty mainly because of the failure to produce an antibody against acid mucopolysaccharides (Humphrey, 1943; Boake and Muir, 1955; Quinn and Cerroni, 1957). Antibodies against the light fraction of protein-polysaccharides extracted from adult mammalian cartilages have, however, been produced by DiFerrante (1964), Loewi and Muir (1965) and Hirschman and Dziewiatkowski (1966). Of these only Hirschman and Dziewiatkowski (1966) coupled the antibody to fluorescein isothiocyanate to use it in histological preparations to detect the relative amounts of protein-polysaccharides in various areas of calcifying cartilage.

A number of animals, particularly embryos, as well as tissues in vitro, have been exposed to an increasing variety of chemicals, e.g. steroid and other hormones, vitamins, trypan blue, papain and thalidomide. The object has been primarily to (a) test the effects of various chemicals intended for therapeutic use, and (b) understand the basic processes of differentiation and morphogenesis by interfering with the normal epigenetic processes. The results to date indicate that such treatments have produced a wide range of effects at a molecular, cytological, as well as gross morphological level.

Landauer (1947) indicated that the extract of adrenal cortex, when injected in chick embryos, causes a retardation in growth. The growth inhibiting effects of various adrenocortical hormones have since been amply confirmed (Sames and Leathem, 1951; Moscona and Karnofsky, 1960). Moreover, Karnofsky, Ridgway and Patterson (1951) and Moscona and Karnofsky (1960) also reported that injections of large doses of cortisone (17-hydroxy 11-dehydrocorticosterone, Kendall's compound E) in chick embryos produces morphological malformations involving eyes, feathers and skeletal tissues. The type and severity of these malformations is dependent on the time and route of cortisone administration. The effects of hydrocortisone (17-hydroxycorticosterone, cortisol, Kendall's compound F) in this respect have been investigated only by Jurand (1968) working on mouse embryos. In the medical field the behaviour of hydrocortisone is to some extent contradictory, for on one hand it is effective against a number of rheumatic and connective tissue diseases and on the other hand, in some cases it delays the repair of cellular injury (see Jurand, 1968). To understand this behaviour of hydrocortisone a considerable amount of work has been done to study the mechanism of its action at molecular level, both on tissues in vitro as well as in vivo (de Duve, Wattiaux and Wibo, 1962; Jacobson, 1964; Kenney, Greenman, Wicks and Albritton, 1965; Polet, 1966; Rancourt and Litwack, 1968). The results from these studies indicate that hydrocortisone has at least two main types of action: first, it increases the DNA-dependent-synthesis of RNA and has a direct controlling effect on the concentration of the specific enzymes, and second, it stabilizes the lipoprotein membranes such as those of lysosomes and red blood cells.

This work reports electron and light microscopic studies of embryonic cartilage of chick and mouse differentiating in vivo and the cartilage of chick differentiated in tissue culture. The work is supplemented by autoradiographic

studies related to the synthesis and secretion of collagen by cartilage differentiated in tissue culture, and immunological studies on the possibility of producing antibody against embryonic chick cartilage. In addition the effects of hydrocortisone on developing limb-bud mesoderm of chick has been studied.

MATERIAL AND METHODS

1. MATERIAL

Chick embryos of Brown Leghorn variety were incubated for varying times in a humid atmosphere at 38°C and were staged according to Hamburger and Hamilton (1951).

Mouse embryos of varying ages were obtained from matings of 6 to 9 week old virgin females with their cousins of JBT strain : the strain has been selected for genes a, b and bt and inbred for approximately 20 generations by brother-sister litter-mate matings and retention of sublines (Jurand, 1965). The embryo was considered as 1-day-old on the day the vaginal plug was found. The embryos were dissected open in the uterus while the pregnant female was under anaesthesia.

Rabbits, used for producing antibody against cartilage, were females of Edinburgh AD or HS stock with a genotype of aa BB DD RR and were about 10 months old, when the experiments started.

2. METHODS

A. Electron microscopy

The whole embryos, but in the case of large embryos only excised hind limb-buds, or cartilage nodules developed in tissue culture were immersed into fixative chilled to 0 - 4°C. Further dissection of the material, if necessary, was carried out in the fixative or in 70% alcohol, depending on the size of material; the fixation of fully-formed cartilage was improved by the prior removal of all the surrounding tissues, including perichondrium. The cartilage was taken from the third toe of the hind limb-bud, or its presumptive area, in chick and mouse *in vivo* studies.

Osmium tetroxide, (OsO_4) 1 or 2% in veronal buffer, (Goel and Jurand, 1968) and glutaraldehyde, 2.5 or 6.5% in sodium phosphate or Millonig's buffer or Pannet-Compton solution, followed by postosmication were tried as fixatives. Usually fixation in 2% OsO_4 in veronal buffer for $\frac{3}{4}$ to 1 hour gave the overall best results. The buffer for OsO_4 contained 1.8% sucrose and 0.1% dried calcium chloride for a more balanced osmotic pressure (Caulfield, 1957) and better preservation of cellular membranes (Palay, McGee-Russell, Gordon, and Grillo, 1962) and extracellular phase (Pearse, 1960). With fully-formed cartilage better results were achieved by fixation in 2.5% glutaraldehyde, in sodium phosphate buffer for $3\frac{1}{2}$ to 4 hours, followed by postosmication in 2% OsO_4, in veronal buffer for 1 hour (see Sabatini, Bensch and Barrnett, 1963). The prolonged washing of the material in the buffer solution after glutaraldehyde fixation and before postosmication, as recommended by Sabatini et al. (1963), did not produce improved end results as compared to the material directly transferred from the glutaraldehyde to OsO_4 solution.

After a thorough wash in stock solution of buffer (5 to 10 minutes, 2 changes) the material was gradually dehydrated in 35% (10 to 15 minutes), 70% (15 minutes), 90% (15 minutes), and 100% (4 changes of 15 minutes each) alcohol; except the last change in 100% alcohol, which was at room temperature, all the previous handling of the material was done at 0 - 4°C. After draining excessive alcohol, the material was transferred directly to the final mixture of Araldite epoxy- resin (Glauert and Glauert, 1958), and was immediately submerged in Araldite to avoid drying of the tissues. Three changes of 45 minutes each were given in Araldite; all the time the material was gently stirred at 45 to 50°C using a slow rotary shaker (Jurand and Ireland, 1965), to enhance the penetration of the viscous epoxy-resin. In the meantime, gelatin capsules were filled $^5/_6$ of their volume with the final mixture of Araldite, and the resin then partly

polymerised in oven, at 54°C, for approximately 4 hours, to prevent sinking of the material to the bottom of the capsule. The material was embedded on the top of the capsule next morning, and was oriented, as desired, before the capsule was left for overnight polymerisation at 54°C.

The glass knives were prepared either by the use of pliers from ordinary glass strips or by LKB knife maker (Type 7801 A) from its standard glass strips. It is worth noting that knives could be stored in a desiccator, without any deteriorating effects up to a period of 3 months. For normal viewing silver to gold coloured sections (c. 700 - 900 Å thick) were cut on a Porter-Blum ultramicrotome and mounted on collodion-carbon-coated copper grids. For autoradiography, gold coloured sections and nickel grids were used.

The sections were stained by floating the grid face downwards on one of the following stains: 2.5% uranyl acetate dissolved in 1% potassium permanaganate (20 minutes; Jurand, 1965), lead citrate (15 - 30 minutes; Reynolds, 1963), or double staining with saturated aqueous solution of uranyl acetate or 20% uranyl nitrate (15 minutes) followed by lead citrate (15 minutes; Saito and Matsunaga, 1966). The latter double staining was routinely used except in early parts of the study. To remove the excessive stain a water jet was used for uranium salts, 0.02N sodium hydroxide jet followed by water jet for lead citrate, and floating the grids on 0.5% citric acid solution (30 seconds) and water for uranyl acetate in potassium permanaganate. Sections for studying the substructure of glycogen granule were, prior to staining with lead citrate, treated with 0.2% periodic acid for 20 minutes (prepared from 50% w/w $HIO_4 . 2H_2O$, BDH; Perry, 1967).

Sections were viewed under an AEI EM 6 or Philips 75 electron microscopes, at settings of 60 kV and 75 kV respectively. The original photographs, taken

on Ilford N 50 plates at a magnification of 1.5 to 40 thousands, were photographically enlarged X2 to X3.

B. Light microscopy

The light microscopic observations were made on paraffin as well as 1μ thick Araldite sections.

For paraffin sections, the embryos were fixed either in 2.5% glutaraldehyde in sodium phosphate buffer (4 hours at 0 - 4°C) or in trichloro-acetic acid-lanthanum acetate, TCA -LA, (4 hours at room temperature; Jurand, 1965) or in 2.5% glutaraldehyde (1 hour) followed by TCA-LA (3 hours). The fixation in glutaraldehyde, as compared to that in TCA-LA, is useful for two reasons: first, it allows a more effective methyl green-pyronin staining of sections and second, the embryos hardly, if at all, change their colour during fixation in glutaraldehyde (the embryos become white opaque within 5 minutes of their immersion in TCA-LA), and therefore whole embryos showing haemorrhage can be photographed on a panchromatic or colour film. The TCA-LA fixation, on the other hand, is preferable to glutaraldehyde fixation for studying the distribution of cells undergoing necrosis or mitosis. After TCA-LA fixation and methyl green-pyronin staining, such cells stain purple, as compared to dull dark green after glutaraldehyde fixation, and are very conspicuous. Double fixation, glutaraldehyde followed by TCA-LA, is preferable for general study because it permits the photography of whole embryos during the early part of the fixation, and also renders the necrotic cells fairly conspicuous, as compared to those in material fixed in glutaraldehyde only.

The embryos were dehydrated in graded alcohols, 70% (30 minutes), 95% (30 minutes), 100% (3 changes of 30 minutes each) and cleared in methyl benzoate (3 changes, material left overnight in last one). Leaving the materials in

any of the alcohols for much longer periods, adversely affects the methyl green-pyronin staining of the sections. The embryos were embedded in 54°C paraffin wax containing little amounts of bees wax and white ceresin (from BDH). Sections 6 μ thick, were cut on a Cambridge rotary rocking microtome. Hydrated sections were stained with methyl green-pyronin (for 1 hour) prepared according to the following formula:

sodium acetate	6.84 mg
hydrochloric acid 1 N	20.00 ml
chloroform purified methyl green crystals	2.20 g
pyronin Y	2.50 g
water	up to 1.00 l

Individual slides were dehydrated and cleared through 95% alcohol (5 seconds), 100% alcohol (5 seconds), 50:50 mixture of 100% alcohol and xylol (10 seconds) and xylol (60 seconds), and mounted in Canada Balsam. For cytochemical work dealing with RNA the hydrated sections were treated with 1 mg/ml RNAse (4X crystallised, salt free, protease free, 40 - 50 Kunitz units/mg activity, dissolved in glass distilled water; from BDH) for up to 3 hours at 38°C before staining with methyl green-pyronin. Control sections were similarly treated with glass distilled water only. Incubation for $1^{3}/_{4}$ hours was only partially effective in extraction of RNA (see Appendix II).

Araldite blocks prepared for electron microscopy were also used for light microscopy. Sections, c. 1 μ thick, were cut on a Porter-Blum Ultramicrotome, collected with the help of a hair loop and placed on a glass slide over a drop of water containing 1% albumen. These sections were routinely stained with 0.5% toluidine blue (in 1% solution of sodium tetraborate) at 38°C for 15 minutes.

For demonstration of glycogen these sections were stained with Periodic acid-Schiff reagent (Schiff's reagent according to Berger and DeLamater; Pearse, 1960); the staining was done at 38°C (30 minutes in 0.5% aqueous periodic acid and 15 - 30 minutes in Schiff's reagent). Treatment of sections with xylol, prior to staining, as suggested by Munger (1961) for staining at room temperature, was not found necessary at 38°C. The sections were counterstained with toluidine blue for general cytoplasm. For cytochemical work concerning glycogen the sections were treated with 2% amylase (2 X crystallised from hog pancreas, minimum activity 500 units/mg; from Worthington Biochemical Co., Freehold, New Jersey) for 2 to 3 hours at 38°C. As the treatment tends to detach the sections from the slides, care needs to be taken after amylase digestion. Digestion of glycogen with saliva was found ineffective.

The sections were viewed and photographed under a Carl-Zeiss Ultraphot II or Wild microscopes. For black and white photography Ilford R 40 panchromatic plates (with various filters to increase the contrast) and for colour photography Kodacolor X film (with Wratten 80 B filter) were used. The magnification of negatives ranged from X 32.5 to X 1,800 and of photographic enlargements up to X3.

C. Tissue culture

Hind limb-buds from 4 to 4½ day old embryos were used; all operations were carried out under asceptic conditions. The excised buds were washed twice in Hank's balanced salt solution (Paul, 1959) and twice in calcium- and magnesium-free balanced salt solution (CMF; Moscona, 1961) and incubated with CMF for 15 minutes at 38°C. This was followed by further incubation for 7 - 10 minutes in CMF containing 1% of trypsin (crystallised and lyopholised, from Worthington Biochemical Co. : minimum activity 150 units/mg, I.U.B. system). The buds were then carefully washed in Hank's balanced salt solution and transferred to standard culture medium, which consisted of Minimal Essential Medium of Eagle (with Earle's

salts) supplemented with 10% horse serum (Flow Laboratories, Irvine, Scotland) and 10 - 50% fresh chick embryo extract (from 8 to 10 day old chick embryos in 50:50 Tyrode's solution: Paul, 1959). The ectodermal jacket was removed with the help of tungesten needles and chondrogenic mesoderm was cut into small pieces, approximately 0.75 mm^3, with cataract knives or dispersed into almost single cells by passing through a micropipette. Suspension cultures (in plastic petri dishes from Falcon Plastics) or hanging drop cultures (using depression slides with a 25 mm wide depression) were set up and culture medium changed every 48 hours, otherwise degenerative changes ensue in the tissue. The suspension cultures were put in a desiccator with 5% CO_2 and 95% air mixture. The hanging drop cultures could not be gassed and care was therefore taken not to expose the culture medium to the atmosphere till it was placed on the cover glass and after that the cover glass was quickly inverted and sealed with wax: this way the pH of the medium remained slightly acidic (6.8 - 7.0). The cultures were incubated up to 6 days at $37.5 \pm 0.5^{\circ}C$.

Cartilage nodules developed in about 40 to 48 hours (see also Jackson, 1965), but the study does not establish any definite time sequence, as regards the various stages of differentiation of the limb-bud mesenchyme into chondroblasts. In the study of Fell (1928), when limb mesenchyme from 3-day-old chicken embryos was cultivated by the coverslip method, it took nearly 6 days to develop a cartilage nodule. The most probable explanation of it seems to be the outgrowth of the cells on the flat surface in the study of Fell (1928) and the lack of such growth in the hanging drop culture in the present study.

The processes of fixation and preparation of the material for microscopic examination were similar to those used for studies of cartilage differentiated in vivo.

D. Autoradiography

Cartilage nodules used in these experiments were allowed to differentiate in tissue culture for two days. The radioactive amino acid, proline-H^3, was used as a precursor for localisation of collagen synthesis and secretion, because of the relatively high content of proline and its hydroxylated derivative, hydroxyproline, in the collagen protein (Revel and Hay, 1963). At the start of the experiment the cartilage nodules were transferred into a petri dish containing culture medium with 15 µc/ml of proline-H^3 (specific activity 720 mc/mM; Radiochemical Centre, Amersham). The cultures were then incubated as before until needed for fixation. The time interval between the onset of the experiment and fixation in various cases was as follows: 15 min., 30 min., 60 min., 2 hours, 4 hours and 24 hours. The procedure from fixation in 2% OsO_4 to collecting the Araldite sections on slides or grids for light and electron microscopy, respectively, was as described for other tissues in appropriate sections; however, the slides used to mount sections for light microscope autoradiography were subbed with gelatin.

For light microscope autoradiography the slides were coated with Kodak AR 10 stripping films and placed in light tight boxes containing Drierite (Anhydrous $CaSO_4$ from Hammond Drierite, Xenia, Ohio) at 0 - 4°C for exposure; procedural details, including subbing of the slides, are given in the leaflet that accompanies the stripping film. For electron microscope autoradiography the grids mounted with sections, were attached to glass slides and coated with a monolayer film of Ilford L-4 emulsion by the method of Caro and van Tubergen (1962), and stored and exposed in the same way as the slides for light autoradiography.

The autoradiographs, whenever needed, were developed in D-19b Kodak developer (4 min.), washed in distilled water (30 seconds) and fixed in Johnson's

Fixsol (10 min.); all the processes were carried out at a temperature of 20± 1°C. The sections were then washed in running tap water for ½ to 1 hour. Staining of light and electron microscopic sections was done in 0.5% toluidine blue and uranyl acetate-lead citrate respectively. It must however be mentioned that with toluidine blue the film was also intensely stained so that the sections became almost invisible; this difficulty was overcome by mounting the sections under Canada Balsam, since the xylol content of the mounting medium preferentially removes the stain from the film, leaving the sections stained. It may be noted that if uranyl nitrate instead of uranyl acetate was used for staining the electron microscope autoradiographs, a black precipitate appeared in the staining solution.

The exposure time required to produce reasonable light microscope autoradiographs of materials incubated for 1, 2, 4 and 24 hours in labelled proline was 40, 20, 15 and 3 days respectively; autoradiographs from cartilage incubated with proline-H^3 for 15 and 30 minutes could not be obtained even after an exposure of 3 to 4 months, which is regarded as the maximum useful exposure time for light autoradiographs using AR 10 films. Nichols (1965) working with bone fragments in vitro also reports: "Two hours of incubation were selected as close to the minimum time at which labelled proline could be found in the collagen of the bone fragments." The exposure time for light microscope autoradiographs also suggested the approximate range of exposure time required for electron microscope autoradiographs: these times are usually X5 as much.

E. Immunological work

The antigen was prepared from limb cartilages of 12-day-old chick embryos. The cartilage was cleaned of all surrounding tissues and central ossifying area of diaphysis, while kept immersed in physiological saline (0.8% sodium chloride).

The cleaned cartilage was ground in a mortar and homogenised in a glass homogeniser, in saline medium. The resultant liquid was centrifuged and only the supernatant, containing saline soluble component of the cartilage, was used in the experiments. It was concentrated by filtration through Sephadex (G 25 coarse, from Pharmacia, Uppsala) until the amount of antigenic solids in the solution was in the range of 5 to 15 mg/ml (estimated by refractometer; from Bellingham and Stanley Ltd., London).

Since only small amounts of antigen could be prepared at any one time, it was considered necessary to use Freund's adjuvant (Freund and McDermott, 1942) to stimulate antibody production. On the first day 2 ml of antigen-adjuvant mixture (a ratio of 1:3) was injected subcutaneously between the clavicles in two rabbits; it was followed by another subcutaneous injection of 1 ml of antigen-adjuvant mixture on the 4th day. Both these injections amounted to 15 mg of antigen per rabbit. These injections were followed by a series of four injections of antigen (50 mg of antigen in total) in saline into the marginal ear vein, within the next month. Ten days after the last injections one rabbit was bled from an incision in the marginal ear vein and 15 ml of the blood removed; a maximum of 25 ml of blood was removed at any one time from any rabbit. The serum was separated by allowing the blood to clot for 2 hours at room temperature, decanting the serum and centrifuging it at 2,000 g for 20 minutes. Precipitin ring tests, performed according to the method of Kabat and Mayer (1964) gave negative results. Therefore, four more injections of the antigen (40 mg) were given in the next four weeks. The subsequent bleeding and precipitin reaction produced positive results only with an antigenic concentration of 5 mg/ml. So in the next two weeks 3 more injections (48 mg of antigen) were given to produce a strong antibody titre. Even then the precipitin tests gave positive results only up to an antigenic concentration of 0.15 mg/ml.

This suggests that embryonic chick cartilage is very weakly antigenic. DiFerranti (1964), Loewi and Muir (1965) and Hirschman and Dziewiatkowsky (1966), however, were able to produce an antibody against PP-L fraction of adult mammalian cartilages. At this stage it was decided to bleed the rabbits and do some fluorescent antibody work; the possibility of negative results, however, was not overlooked.

The technique used was largely that recommended by Nairn (1962). Lissamine Rhodamine B (RB 200, from G.T. Gurr) was converted to its sulphonyl chloride. Conjugation of the fluorochrome with the antibody was carried out after separating the globulins from the whole serum by precipitation with 50% saturation of ammonium sulphate. The globulin solution was mixed with twice the volume of carbonate-bicarbonate buffer (pH 9.0; M 0.5) and stirred thoroughly at 0 - 4°C, while the solution of fluorochrome (0.1 ml of fluorochrome; 1.0 ml of globulin) was slowly added over a period of 15 minutes; stirring continued for another half hour. The fluorochrome-antibody complex was separated from any uncoupled fluorochrome by passing it through a Sephadex G 25 column, and was brought to its original volume by dialysing against polyhydroxyl alcohol.

10-day-old chick embryos were used to test the fluorescein-labelled antibody. The embryos were snap frozen by dropping in liquid nitrogen for 5 minutes and after freeze substitution by the usual procedure were embedded in 54°C paraffin wax. The hydrated sections of the embryos containing fully formed cartilage were allowed to react with the fluorescein-labelled antibody for 1 3/4 hours at room temperature, in a humid atmosphere. The sections were washed in several changes of saline, mounted in buffered glycerol and viewed under Reichert Zetopan microscope with an Osram HBO 200 mercury vapour lamp to provide UV illumination. Control sections were allowed to react with fluorescein-

labelled normal serum.

The results indicate a flourescence only in the perichondrial region of the cartilage, but no flourescence in the cartilage matrix.

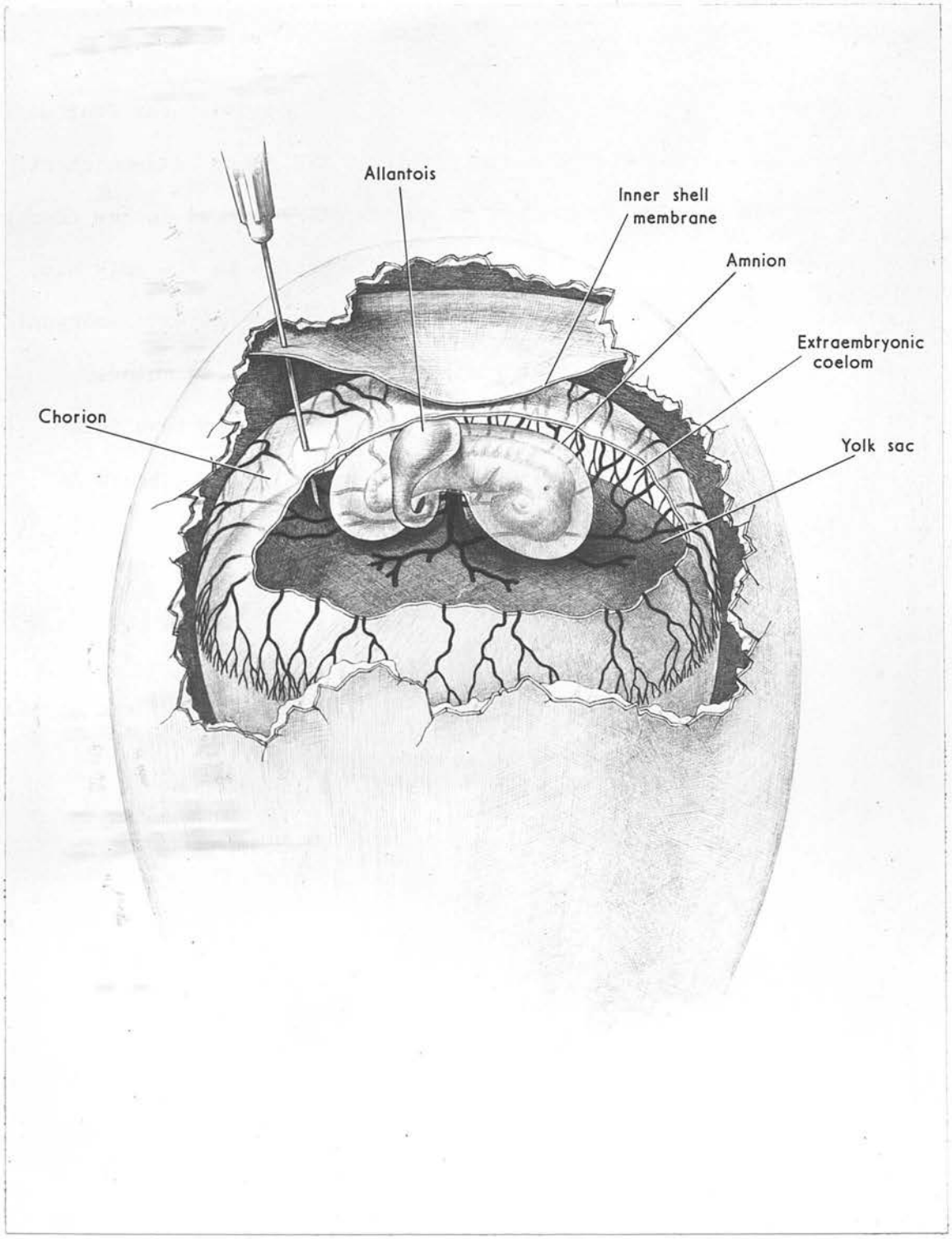
F. Effects of hydrocortisone

In the present experiments the source of hydrocortisone was hydrocortistab injection (Boots Pure Drug Co.), which consists of 25 mg hydrocortisone acetate per ml in an aqueous vehicle containing not more than 1% suspending agents with 0.9% sodium chloride and 0.9% benzyl alcohol. The concentration of hydrocortisone acetate per ml, if necessary, was adjusted by addition of sterile 0.9% sodium chloride solution.

In the earlier experiments 3- and 4-day-old chick embryos were injected with various doses, 1 mg to 10 mg, of hydrocortisone acetate in the yolk sac or in the extraembryonic coelom (see text-figure 1). The results indicated that (a) the injections in the extraembryonic coelom are considerably more effective than those in the yolk sac (see also, Moscona and Karnofsky, 1960), (b) 4-day-olf embryos, when injected with 7.5 mg hydrocortisone/egg (LD 50), in some cases show haemorrhages in one or more limb buds after 24 hours of incubation: LD 50 in these experiments refers to the dose of hydrocortisone which when injected in the extraembryonic coelom of 4-day-old chick embryo kills about 50% of the embryos within the next 24 hours. Injecting the drug in the extraembryonic coelom requires that the embryos must lie just under the air space of the egg; to achieve this the eggs were kept with their broad ends up for half an hour before opening the shell. An injection in the extraembryonic coelom can be easily distinguished from an injection in the yolk sac, because only in the former case the whole surrounding area of the embryo instantaneously shows a milky colour of the injected suspension of hydrocortisone.

Text figure 1

Diagrammatic representation of the chick egg incubated for four days, to show the extraembryonic coelom, that is the space between chorion mesoderm and yolk sac mesoderm. A window has been cut in the chorion to show the following: attachment of the embryo to the yolk sac; amnion; and allantois. The point of needle shows the extraembryonic coelom, the area where hydrocortisone was routinely injected. Allantochorion is in the process of formation. To achieve this orientation of embryo in relation to the egg, it is necessary to hold the egg with its broad end up for half an hour.


Allantois
Inner shell membrane
Amnion
Extraembryonic coelom
Chorion
Yolk sac

After injections the eggs were sealed with a parafilm (from Gallenkamp Ltd.) and replaced in an incubator with forced air circulation and humid atmosphere at 38°C. The control embryos were injected with saline solution.

The eggs were reopened after 24 hours and living embryos fixed in 2.5% glutaraldehyde in sodium phosphate buffer or TCA-LA. The latter fixative was used only for studying the distribution of necrotic cells. The embryos were examined under a stereoscopic microscope (Watson, Barnett) and weighed on a chemical balance. Photographs, whenever necessary, were taken with an Exakta (Varex IIA) camera on a Kodacolor X (with Wratten 80 B filter) or 50 ASA Pan F film, while the embryos were still in glutaraldehyde. The rest of procedures, sectioning and staining, were as usual and are described under procedure for paraffin sections for light microscopes (see p. 19).

RESULTS

1. DIFFERENTIATION OF HIND LIMB-BUD CARTILAGE IN CHICK IN VIVO

I. Light microscopic observations

(a) Mesenchyme (chick, stages 23 and 26). The mesenchyme at stage 23 consists of a mass of loosely-packed, homogeneously-dispersed cells (plate 1, fig. 1). The cell nucleus is very large and usually has only one, but sometimes two, darkly staining nucleoli. The cytoplasm forms a thin layer around the nucleus and the cell membrane is not well-defined. The nucleo-cytoplasmic ratio, that is the total area of nucleus per total area of the cytoplasm is high, approximately between 4 and 5. The extracellular phase is hyaline in appearance. The cells are in contact with each other either by cell-to-cell attachment or through their cytoplasmic extensions.

The cells at stage 26 noticeably differ from those of stage 23 only in being more often connected to each other by cytoplasmic extensions than by cell-to-cell attachment (plate 1, figs. 2 and 3). The mitotic index* of 46 suggests that these cells are actively dividing.

(b) Prechondrogenic tissue (chick, stage 29). This is the earliest stage when the area of future cartilage is histologically detectable in the apical region of the hind limb. The cells that will participate in cartilage formation already give the impression of being concentrically arranged (plate 1, fig. 4). Moreover, these cells, as compared with the rest of mesenchyme cells, show fewer cytoplasmic extensions. A mitotic index of 35 is calculated.

* A mitotic index is normally defined as 'total number of nuclei simultaneously undergoing mitosis per total number of 1,000 nuclei' (Henderson and Henderson, 1963), but for the ease of observation the term is used here to denote the number of nuclei in metaphase or anaphase per total number of 1,000 nuclei.

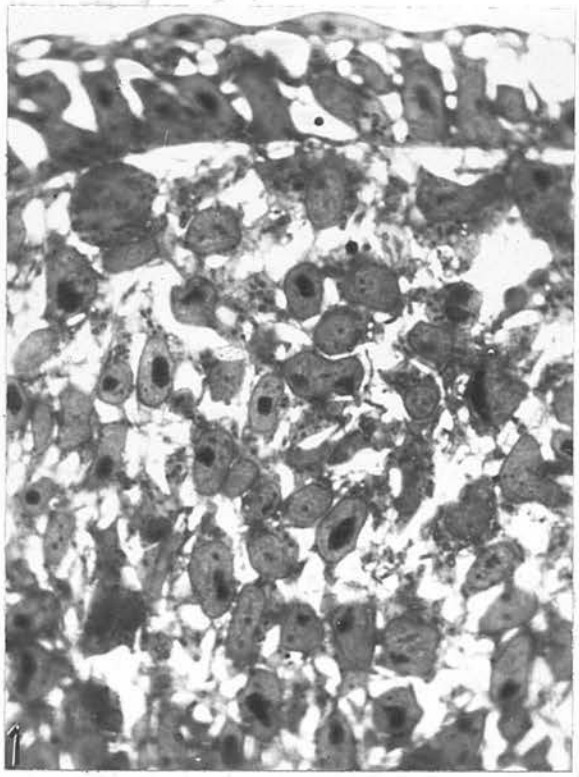
Plate 1.

Figure 1. Mesenchyme (chick, stage 23). Notice the relatively large nuclei, to some extent syncytial appearance of mesenchyme and transparent nature of extracellular phase. The epidermis and basement membrane are towards the top. M1, X 1,100.

Figure 2. Late mesenchyme (chick, stage 26). These undifferentiated cells have frequent intercellular connections. The epidermis is towards the top. M3, X 250.

Figure 3. Late mesenchyme (chick, stage 26). Same as figure 2, but under high power. See frequent intercellular connections, and also mitoses (arrows). M3, X 1,000.

Figure 4. Prechondrogenic tissue (chick, stage 29). The outline and arrangement of the centrally placed cells - the future chondroblasts, is noticeably different from those of mesenchyme cells placed near the periphery of micrograph. M1, X 400.



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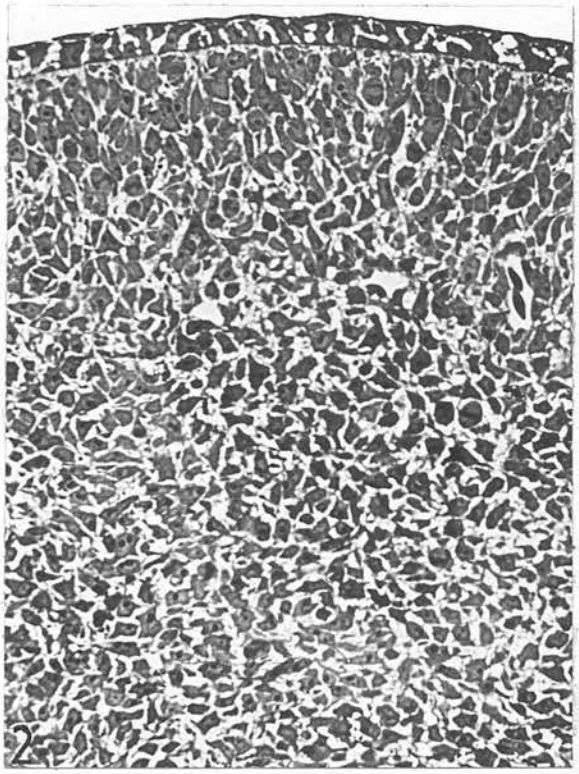

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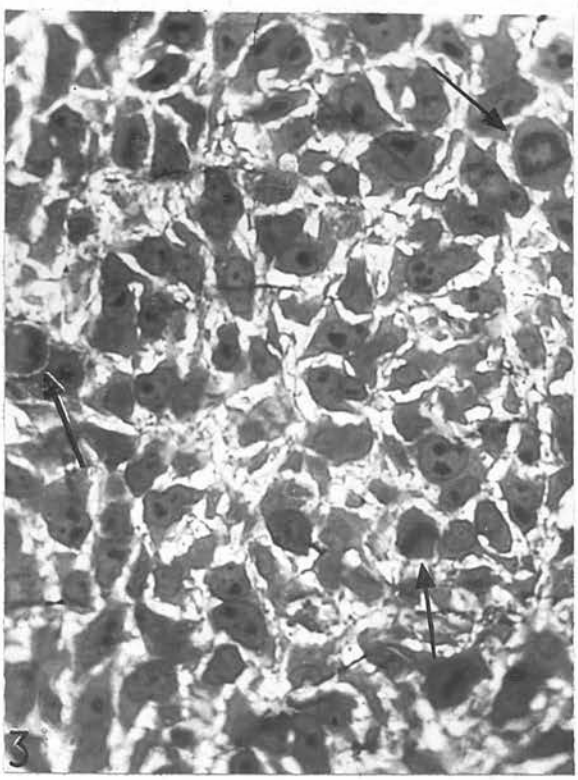

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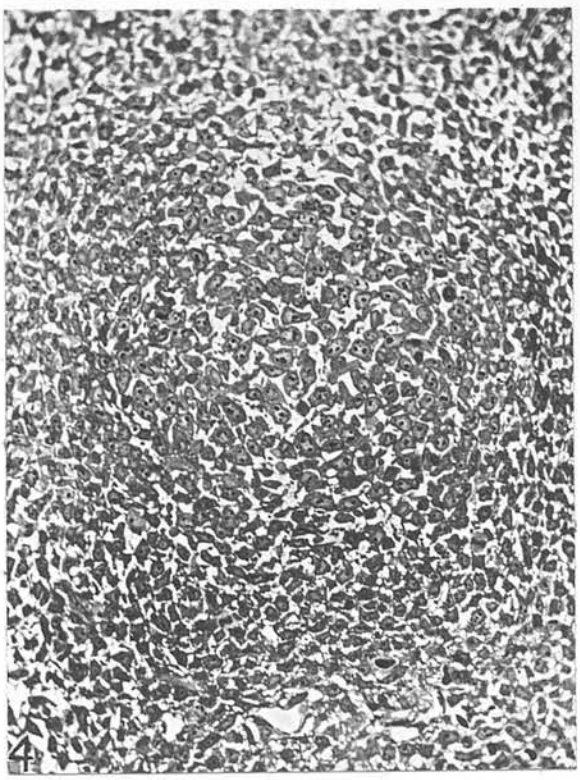

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(c) Chondrogenic tissue (chick, stage 31). The area of future cartilage is now very well marked and is usually termed a 'condensation'. The cells towards the periphery of the condensation are thin and long in appearance and concentric in arrangement (plate 2, fig. 1). The cells in the centre of the condensation are rounded and with very few cytoplasmic extensions. The nucleo-cytoplasmic ratio, approximately 1.5, is appreciably lower, as compared to that of stage 23. The presence of a small amount of extracellular matrix is evidenced by a faint metachromatic stain after toluidine blue staining. There is considerably more extracellular space as compared to stage 29. The mitotic figures are less frequent towards the centre of the condensation (mitotic index 20) than at the periphery (mitotic index 30).

(d) Late chondrogenic tissue (chick, stage 37). The cartilage tissue is now distinct from the perichondrium but there is an intermediate zone consisting of one layer of cells (plate 2, fig. 2). The perichondrium consists of 4 to 5 layers of thin, elongated and closely-packed cells with centrally placed nuclei. The chondroblasts are rounded and only rarely have any intercellular connections (plate 2, fig. 3). The eccentrically placed nucleus is relatively small and is lightly stained as compared to the cytoplasm, which sometimes shows vacuolar areas, due to the presence of the Golgi apparatus. The highly vacuolated appearance of these cells is also seen in methyl green-pyronin stained sections of the material prepared by glutaraldehyde fixation and paraffin embedding. Toluidine blue stained Araldite sections show a large amount of metachromatically stained extracellular fibrous matrix. No lacunae are seen around the chondroblasts and mitotic figures are rarely encountered at this stage.

(e) Epiphyseal embryonic cartilage (chick, stage 39). The chondrocytes are sharply separated from the perichondrium (plate 2, fig. 4), and are regular in shape (plate 2, fig. 5), and lack intercellular connections. The nucleo-

Plate 2

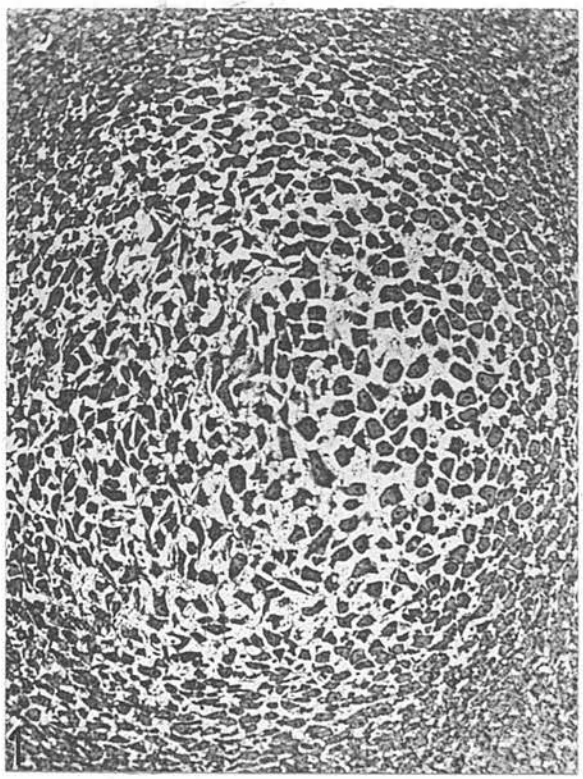
Figure 1. Chondrogenic tissue (chick, stage 31). The 'condensation' is well marked. The cells in the perichondral area are concentrically arranged, while there is a large extracellular phase and fewer intercellular connections in the centre of the condensation. M1, X 270.

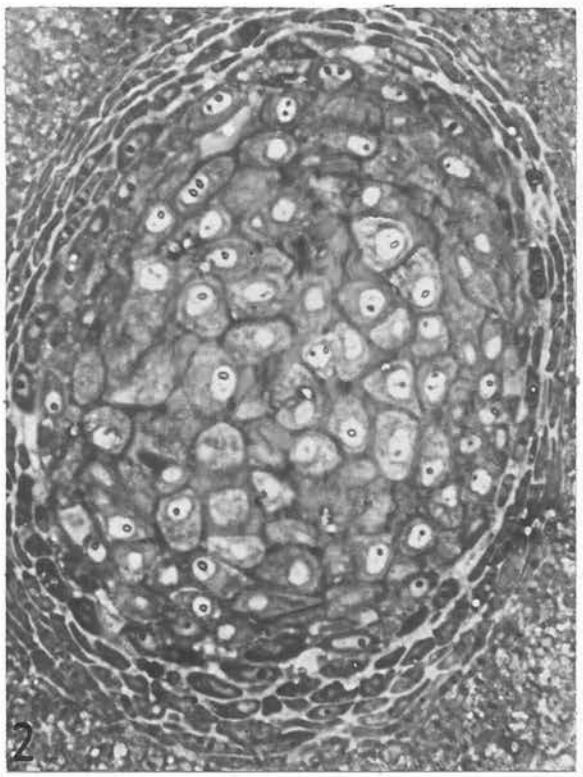
Figure 2. Late chondrogenic tissue (chick, stage 37). Notice a clear distinction between perichondrium and chondroblasts. M1, X 520.

Figure 3. Late chondrogenic tissue (chick, stage 37). Same as figure 2, but under high power. It shows the distinction between the cells of perichondrium (pc), which have centrally placed and elongated nuclei, and the chondroblasts (ch), which have eccentrically located and rounded nuclei. Also note the extensive extracellular phase, and vacuolar area representing the Golgi vacuoles (gv). M1, X 1,200.

Figure 4. Epiphyseal embryonic cartilage (chick, stage 39). A sharp demarcation between the perichondrium and chondrocytes is seen. It also indicates the inefficiency of fixation in osmium tetroxide without prior removal of perichondrium, as evidenced by hyaline appearance of chondrocytes. M1, X 1,60,

Figure 5. Epiphyseal embryonic cartilage (chick, stage 39). Note the extensive extracellular phase, absence of intercellular connections, relatively small nuclei, and even a mitotic figure (arrow). M2, X 1,900.



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gv
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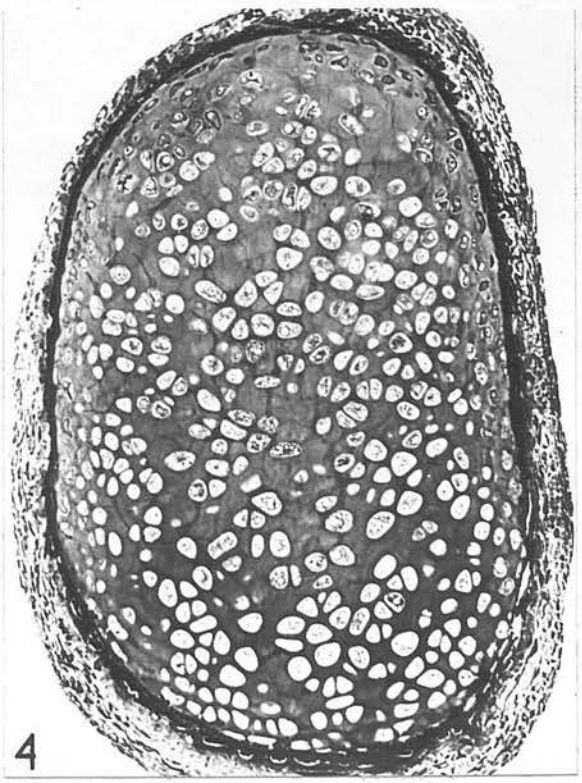
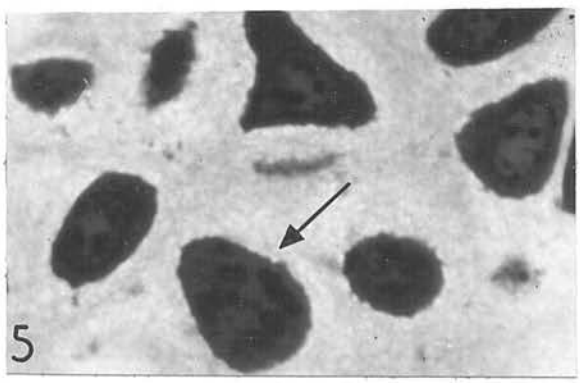




cytoplasmic ratio is around 0.5 as compared to between 4 and 5 in the mesenchyme cells at stage 23. The areas of the Golgi apparatus as well as of the saccular cisternae of the endoplasmic reticulum can usually be identified. The extracellular fibrous matrix is very extensive in amount and shows intense metachromasia with the toluidine blue. Chondrocytes undergoing mitotic divisions are only rarely observed. The periodic acid- Schiff reaction for glycogen gives negative results.

II. Electron microscopic observations

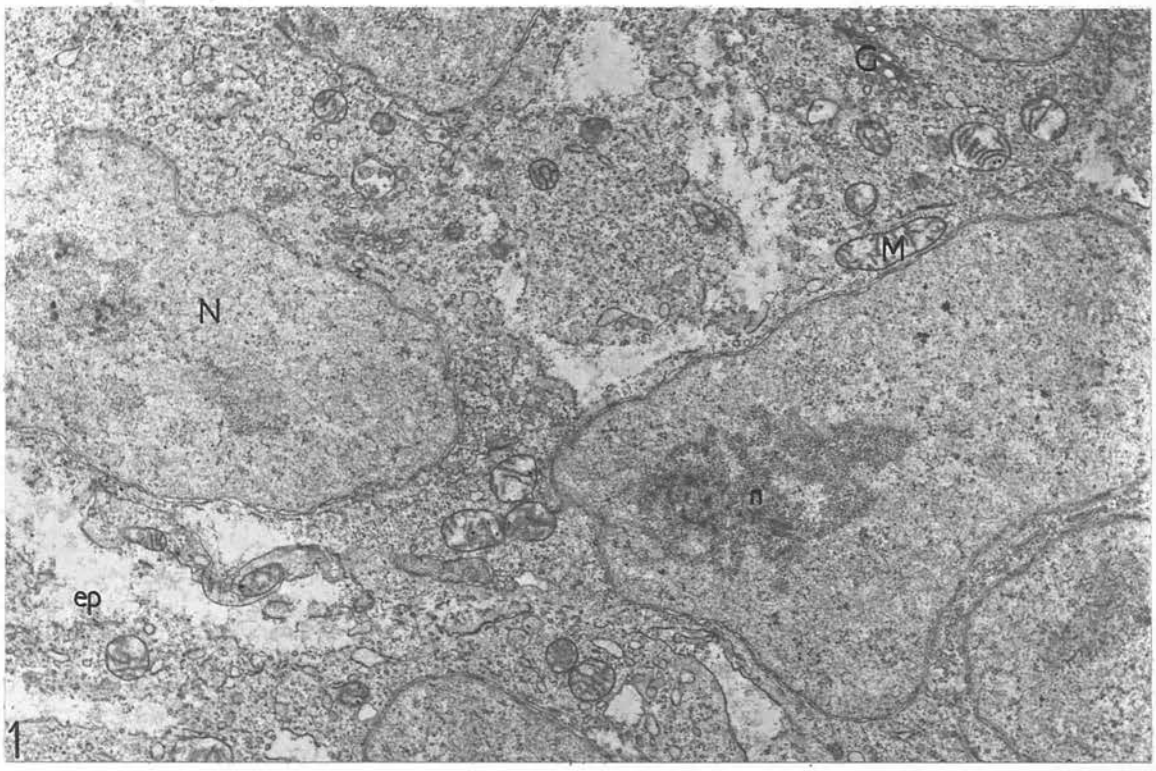
(a) Mesenchyme (chick, stage 23). The undeveloped state of cytoplasmic organelles indicates that the early mesenchymal cells are undifferentiated.

At this stage the nucleus is relatively large and centrally placed (Plate 3, fig. 1). It is surrounded by a nuclear envelope consisting of outer and inner nuclear membranes enclosing the perinuclear cisterna. The thickness of the nuclear envelope, in cross section, varies from 40 - 90 mμ, depending on the width of the perinuclear cisterna. The outer nuclear membrane is sometimes continuous with vesicular profiles, probably of endoplasmic reticulum (plate 3, fig. 2; plate 4, fig. 1). The envelope frequently shows nuclear pores, about 70 mμ across, where the two nuclear membranes are continuous with each other. The pore is bridged by a condensed 'diaphragm' or septum and contains very electron dense material (plate 4, fig. 2). In the nuclear matrix, consisting of fibrillar and granular elements, chromatin can not be distinctly identified, but there are some unevenly dispersed darker granules in the light background (plate 3, fig. 2). These granules are unidentified components of the nuclear matrix. The nucleolus, eccentrically placed inside the nucleus, is interspersed with relatively electron translucent areas which are indistinguishable from the nuclear matrix. The nucleolus consists of a large particulate area, which is moderately electron dense, and small fibrillar areas, which are very electron dense. The particulate area has ribosome-like granules (nucleolind of Love, 1965).

Plate 3

Figure 1. Mesenchyme (chick, stage 23). A low power electron micrograph to show the relatively large and centrally located nuclei (N) and to some extent 'syncytial' appearance of tissue. M1, X 9,000.

Figure 2. Mesenchyme (chick, stage 23). Note the fragmentary nature of the plasmalemma (pl), paucity of the granular endoplasmic reticulum (er), the electron translucent nature of the extracellular phase (ep), and the 'blebbing' of the nuclear envelope (ne). Also see the characteristic particulate area (pn) and fibrillar area (fn) of the nucleolus and some unidentified electron dense granules and chromatin (cr) in the nuclear matrix. M1, X 20,700.



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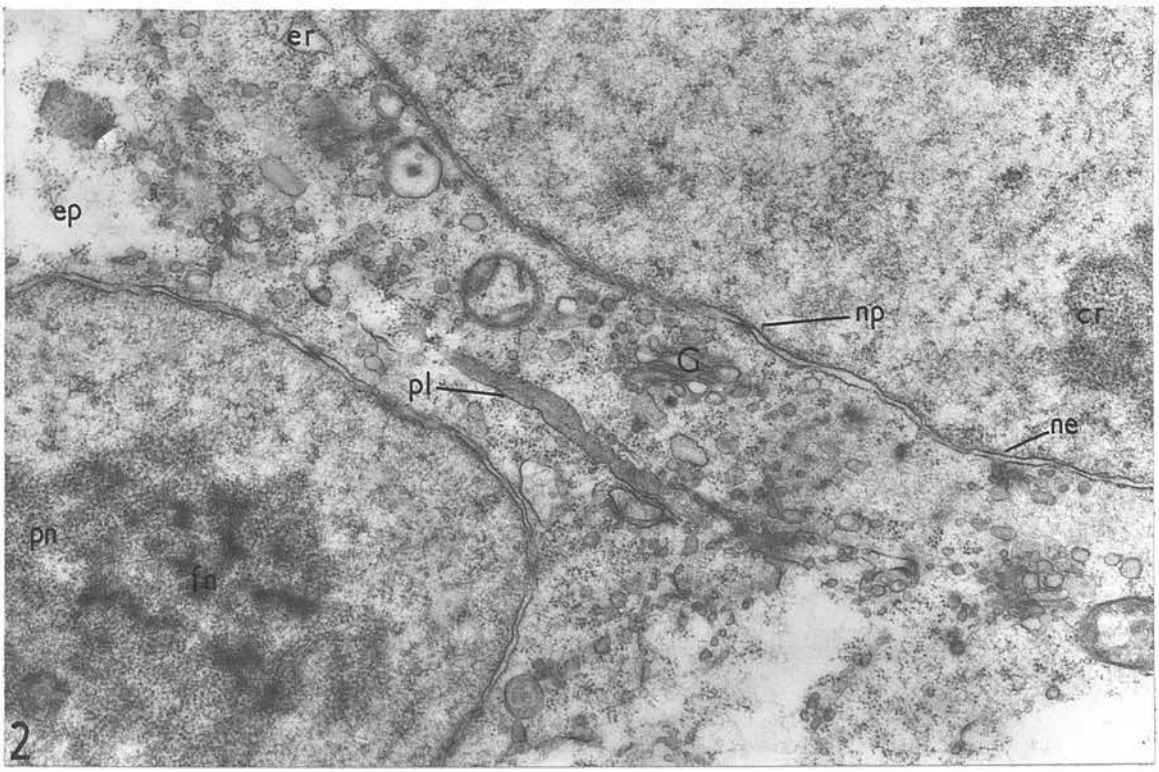


2

The cytoplasmic ground substance is almost completely electron transparent in appearance and contains numerous ribosomes present either singly (free ribosomes) or in groups of polysomes, each with 5 to 8 ribosomes. The only membraneous structures to which they are sometimes attached are the profiles of the endoplasmic reticulum.

The endoplasmic reticulum consists mainly of smooth vesicles usually about 0.1 to 0.4 μ in diameter, but sometimes a few elongated and granular vesicles can also be seen. The contents of the endoplasmic reticulum are amorphous and electron translucent.

The Golgi apparatus is not prominent and is represented, in sections, by one or two groups of 3 to 5 lamellae and a few 0.1 μ across vesicles on either side of the lamellae. The lamellae are dilated towards their ends where their contents are more electron translucent. Cytosomes, that is vacuolar bodies which may contain lipids, vesicles and other unidentified structures, occur infrequently and are usually around 0.5 μ in diameter.

The mitochondria are numerous and appear, in sections, either circular (c. 0.5 μ in diameter) or elongated (up to 1.76 μ x 0.66 μ). The well preserved mitochondrial cristae are 24 to 38 mμ wide. The mitochondrial matrix is mostly electron translucent and contains a few electron dense mitochondrial granules about 34 to 58 mμ in diameter.

The plasmalemma is usually either fragmentary or missing over large areas of cells and the appearance of the mesenchymal tissue is to some extent syncytial.

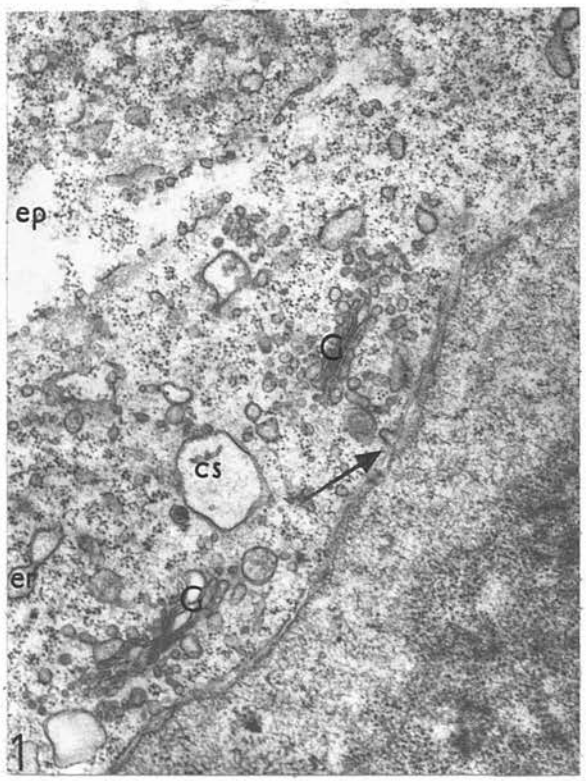
The extracellular phase is electron translucent and is devoid of any fibrous components.

Plate 4

Figure 1. Mesenchyme (chick, stage 23). Notice the blebbing of the outer nuclear membrane (arrow), and the characteristic appearance of Golgi apparatus (G), with few lamellae and vesicles. M1, X 20,700.

Figure 2. Mesenchyme (chick, stage 23). The structure of the nuclear pore (np) with its diaphragm is very clear. Also note the multivesicular bodies (mv) and cytosome (cs). M1, X 20,700.

Figure 3. Late mesenchyme (chick, stage 26). Notice the fairly large, centrally located nuclei (N), electron translucent extracellular phase (ep) and extracellular membraneous structures (em). M3, X 9,000.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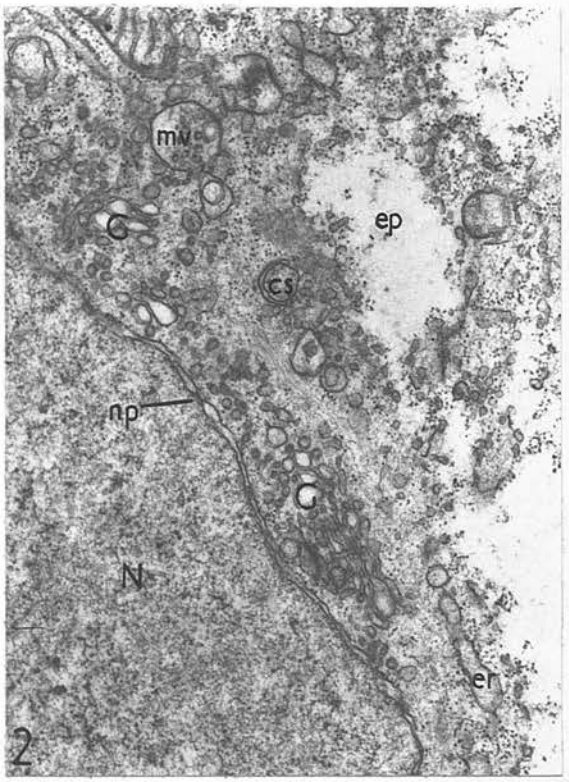
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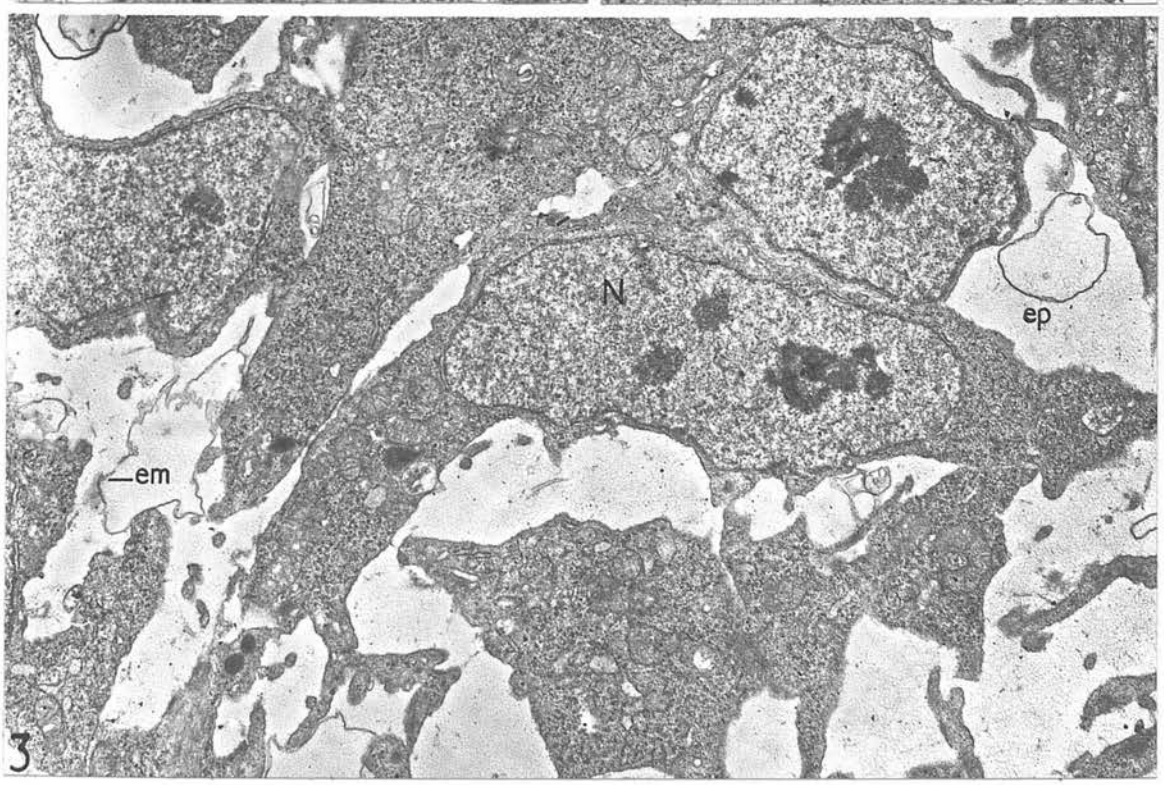
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(b) Late mesenchyme tissue (chick, stage 26). These cells, at the ultrastructural level, show changes that are mainly related to the apparatus concerned with protein synthesis, namely the ribosomes and the endoplasmic reticulum. The ribosomes are present in greater quantity, as compared to those in the stage 23, and are mostly in the form of polysomes. The profiles of the endoplasmic reticulum are frequently elongated and granular in appearance, due to the attached ribosomes. Some vesicular profiles, as seen in the stage 23, are also present (plate 5, fig. 1).

The nucleus is still fairly large and centrally located. The nuclear pores may have a diaphragm but the material inside the pores is less electron dense (plate 5, fig. 2). The outer nuclear membrane occasionally has ribosomes attached to it. Ribosome-like particles, measuring 15 to 20 mμ in diameter, are also present in the nuclear matrix as well as in the nucleolus (plate 5, fig. 3).

The ultrastructure of the juxtanuclear Golgi apparatus does not noticeably differ from that of stage 23. The mitochondrial matrix is moderately electron dense.

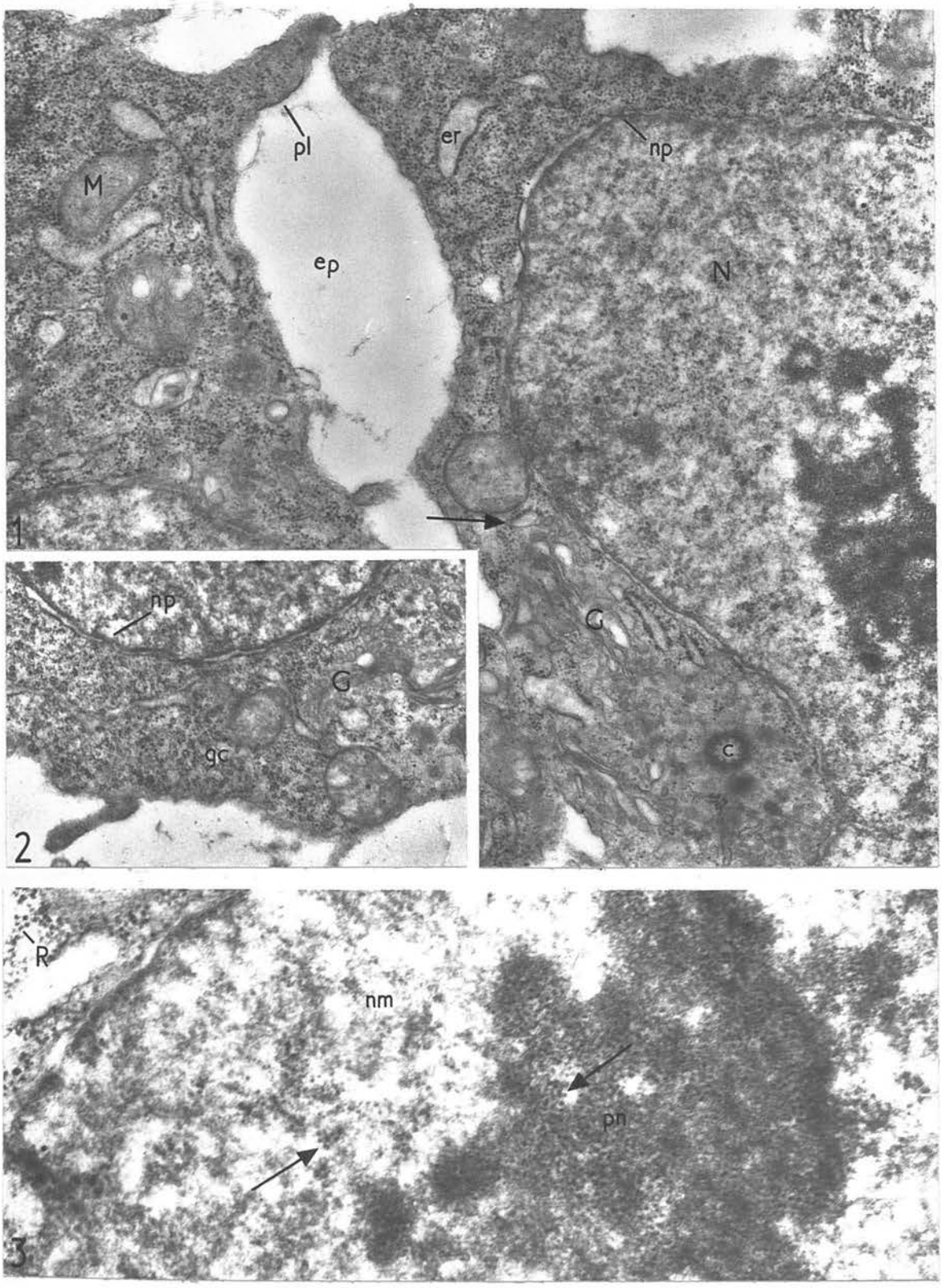
A centriole, in a cross section, is seen in close proximity to the Golgi apparatus in plate 5, figure 1. It is c. 200 mμ in its outer diameter and c. 100 mμ in inner diameter; its interior is occupied by a homogeneous cytoplasm of low electron density. Its wall is made of nine evenly-spaced triplets of tubules. Each triplet, approximately 50 mμ wide and 25 mμ thick, is embedded in an electron dense amorphous matrix. The arrangement of the triplets in the centriolar wall is of a slightly overlapping nature, like a cross section of the blades of a turbine. A spherical appearing pericentriolar satellite, about 100 mμ in diameter, is present as an electron dense mass with an ill-defined boundary. A similar structure of the centriole has been described by Fawcett (1966).

Plate 5

Figure 1. Late mesenchyme (chick, stage 26). This micrograph shows a well defined plasmalemma (pl), elongated profiles of the granular endoplasmic reticulum (er), centriole (c) with its pericentriolar satellite and the electron translucent extracellular phase (ep), The arrow points to a partly granular, vesicular profile of the endoplasmic reticulum. M3, X 31,400.

Figure 2. Late mesenchyme (chick, stage 26). Notice the ground cytoplasm (gc) filled with the polysomes and the nuclear pore (np) containing less electron dense material as compared to one in plate 4, figure 2. M3, X 20,700.

Figure 3. Late mesenchyme (chick, stage 26). A high power electron micrograph to show the similarity between the ribosomes (R) in the cytoplasm, and similar bodies (arrows) inside the particulate area of nucleolus (pn) and nuclear matrix (nm). M3, X 56,000.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The plasmalemma is 11 to 14 mμ thick and fairly well-defined. There are frequent cytoplasmic extensions forming intercellular junctions with similar extensions of the neighbouring cells. Thin membraneous structures, of unknown origin and sometimes connected to the plasmalemma are occasionally observed in the extracellular phase. They are possibly an artifact.

The extracellular phase, like that of stage 23, is electron translucent and without any matrix (plate 4, fig. 3).

(c) Chondrogenic tissue (chick, stage 31). The chondroblasts present an ultrastructural appearance characteristic of cells engaged in synthesis of material for extracellular use; they appear compact, owing to their high electron density. The cells have a scalloped appearance, probably due to the extrusion of vacuoles containing secretary material (plate 6, fig. 1). The extracellular phase is much more extensive than in the previous stages and the intercellular connections are much less frequent.

The nuclei are small as compared to those of the earlier stages, and the external nuclear membrane is sometimes continuous with vesicular profiles, which probably belong to the endoplasmic reticulum system (plate 7, fig. 2; plate 8, fig. 1).

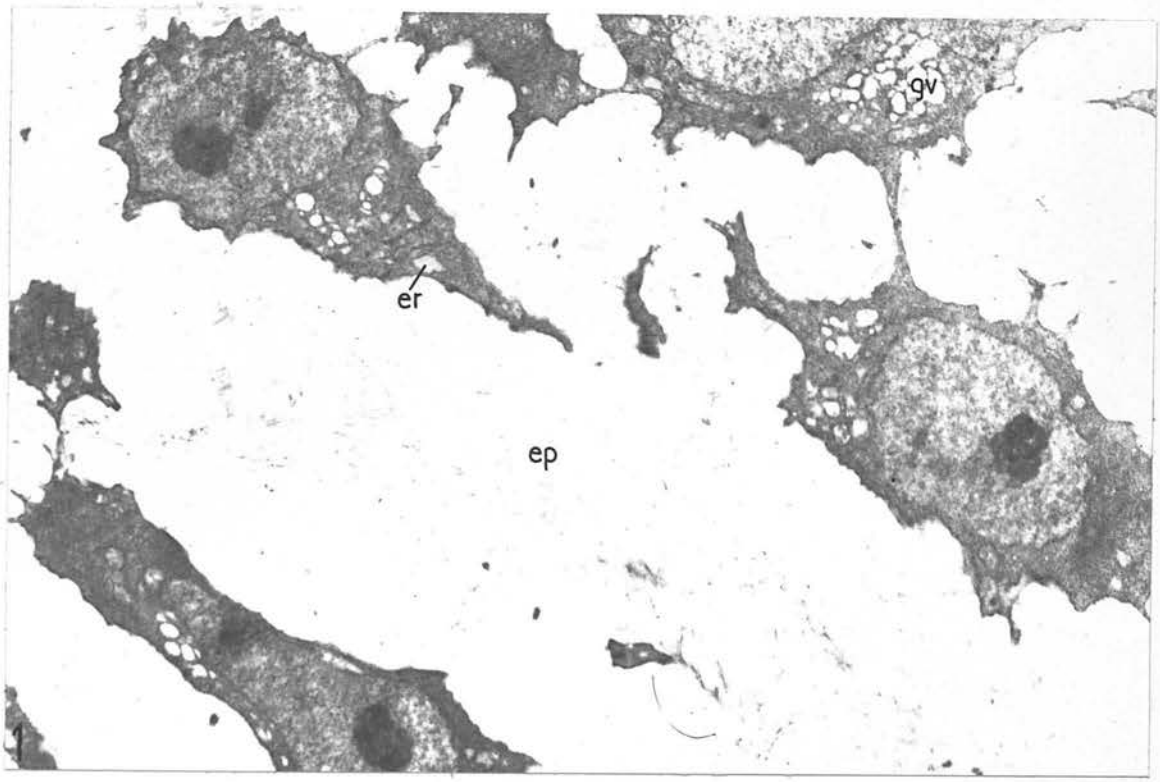
The endoplasmic reticulum is well developed and consists mainly of interconnected elongated cisternal profiles around 60 to 100 mμ across. These profiles are often dilated, forming saccular cisternae of various sizes; some as large as 0.3 μ across and 0.7 μ long (plate 7, fig. 1). The saccular cisternae are never regular in their outline. Sometimes they are seen to reach or fuse with the plasmalemma, but seldom contact with the extracellular phase as seen in plate 7, figure 1. The vesicular profiles of the reticulum measure around 100 - 200 mμ in diameter. Sometimes their walls are also fused with the plasmalemma (plate 7, figs. 2 and 3) or they are in contact with the extracellular phase (plate 7, fig. 3).

Plate 6

Figure 1. Chondrogenic tissue (chick, stage 31). A low power electron micrograph to show a large extracellular phase (ep) with some amount of matrix in it; the characteristic scalloped appearance of chondroblasts, and a clear distinction between the hyaline of the Golgi vacuoles (gv) and the saccular cisternae of the endoplasmic reticulum (er). M5, X 6,300.

Figure 2. Chondrogenic tissue (chick, stage 31). This part of chondroblast shows the formation of the Golgi vacuoles (gv) by adlineation and coalescence of smaller vacuoles and vesicles, and relationship of extracellular fibres (f) with plasmalemma (pl) at a point where cell seems to secrete its product by merocrine secretion. Also note the difference between the Golgi vacuoles (gv) and the saccular cisternae of the endoplasmic reticulum (er). M5, X 23,200.

Figure 3. Chondrogenic tissue (chick, stage 31). It shows the relationship between the extracellular fibres (f) and the plasmalemma (pl), and it appears that former 'sprouts' from the latter. M5, X 27,800.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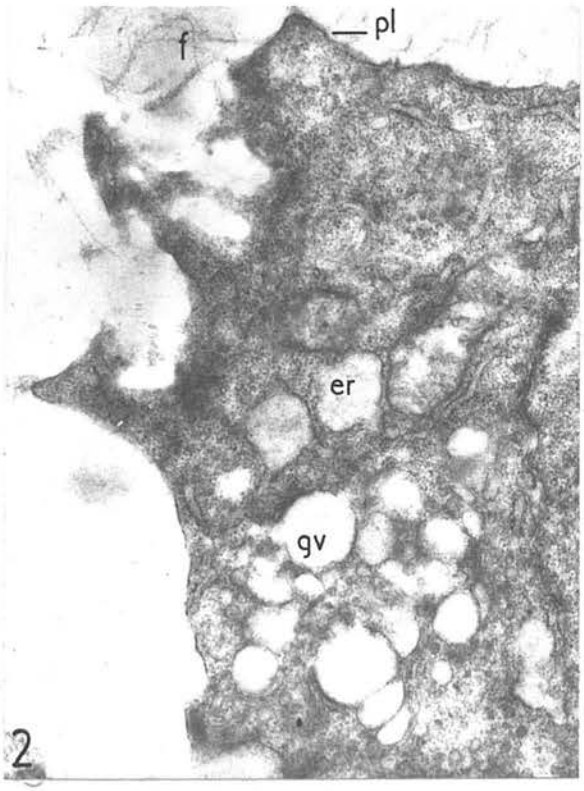
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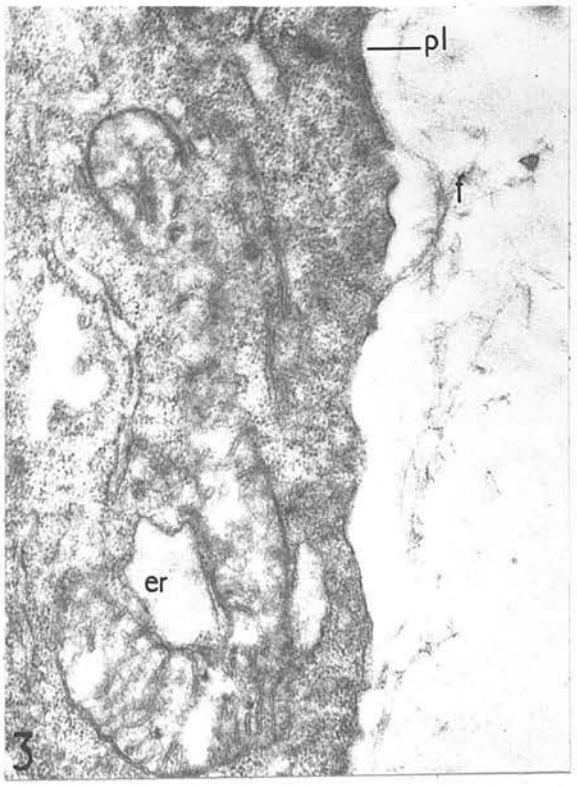
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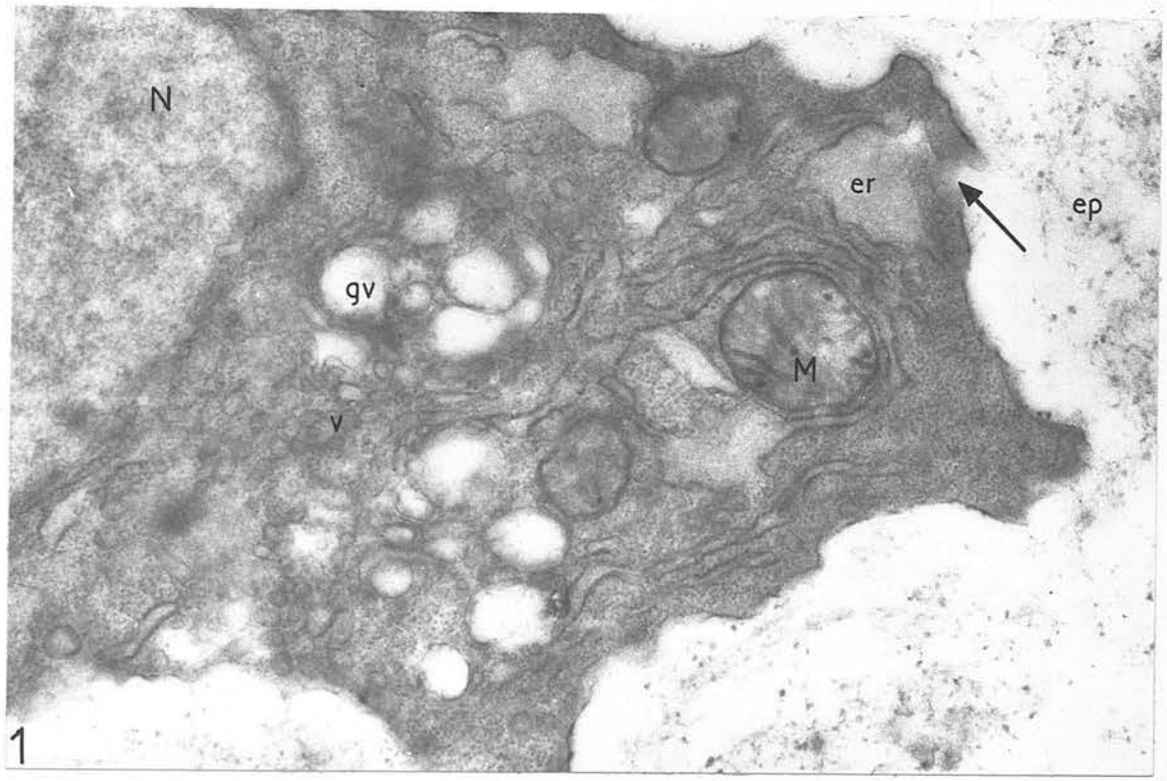
Plate 7

Figure 1. Chondrogenic tissue (chick, stage 31). It shows a direct communication (arrow) between one of the cisternae of the endoplasmic reticulum (er) and the extracellular phase (ep). Also note the difference between the saccular cisternae of the endoplasmic reticulum (er) and the Golgi vacuoles (gv) and formation of latter by adlineation and coalescence of smaller vacuoles and vesicles (v). M5, X 27,800.

Figure 2. Chondrogenic tissue (chick, stage 31). This high power micrograph shows adlineation and fusion of the vesicular profiles (arrows) with the plasmalemma (pl), and also 'blebbing' of nuclear envelope (ne), near which there is a nuclear pore (np). M1, X 40,600.

Figure 3. Chondrogenic tissue (chick, stage 31). A high power electron micrograph showing one vesicular profile fusing with the plasmalemma, and another communicating with the extracellular phase (arrows). M1, X 72,400.



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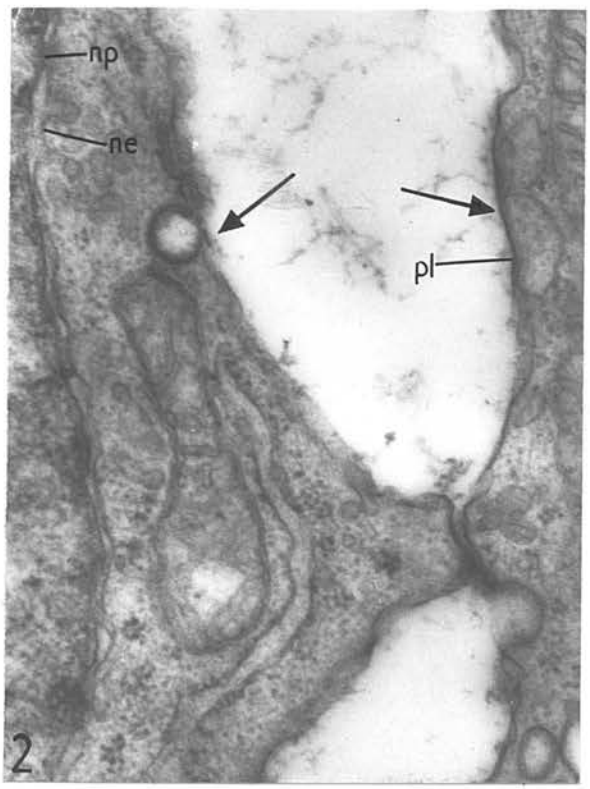

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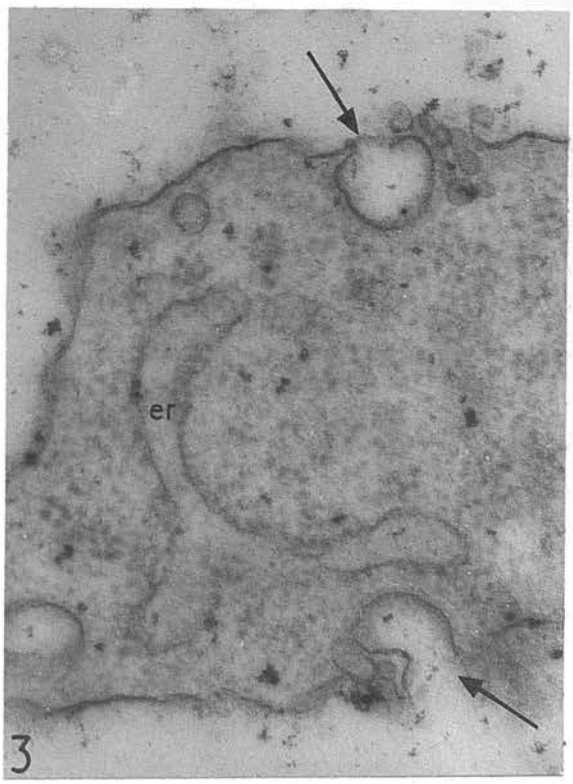

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The endoplasmic reticular system is filled with an amorphous, moderately electron dense material and its surface membranes are almost always studded with ribosomes. The ribosomes are also present as free polysomal groups.

The Golgi apparatus in this stage is the most conspicuous cell organelle and consists of one or two groups of lamellae, numerous small vesicles and a few vacuoles. The vesicles are usually oval or circular, smooth-membraned sacs about 70 to 80 mμ in diameter and are at times indistinguishable from the vesicles of the endoplasmic reticulum. Moreover, the Golgi vesicles seem to contribute to the formation of the Golgi lamellae (plate 8, fig. 1). In plate 8, figure 2 a continuity between a smooth lamella of the Golgi apparatus and a granular endoplasmic reticulum cisterna is clearly noticeable. The Golgi lamellae as well as the vesicles contain a moderately electron dense amorphous material similar to that in the endoplasmic reticulum. The Golgi vacuoles, on the other hand, are large and usually rounded structures with electron translucent contents. They are seen towards the maturing phase of the Golgi apparatus and seem to be derived either from the swelling of the Golgi lamella (plate 8, fig. 1) or by enlargement and fusion of small vesicles (plate 7, fig. 1; plate,8, fig. 2). The electron transparency of the contents of the vacuoles is proportional to their size.

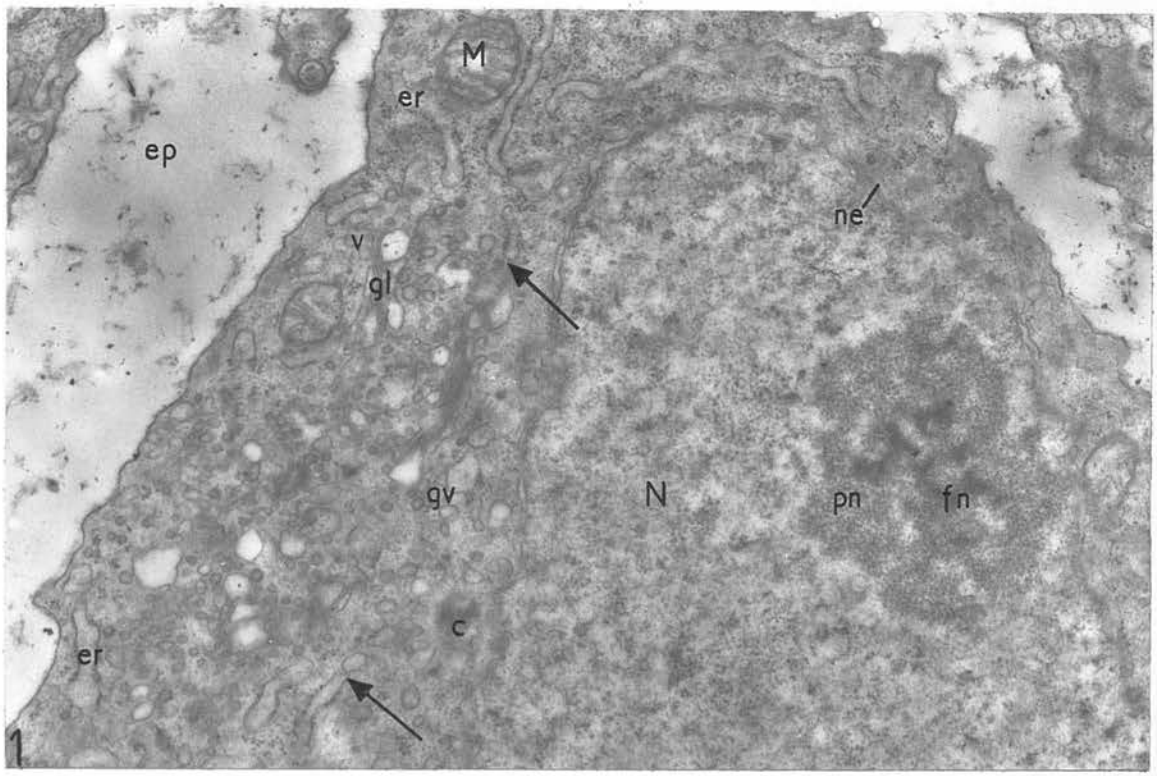
The differences between the Golgi vacuoles and the saccular cisternae of the endoplasmic reticulum are as follows: first, the contents of the Golgi vacuoles are electron translucent while those of the cisternae are homogeneous and moderately electron dense; second, the Golgi vacuoles are regular in outline while the cisternae are not; third, the limiting membranes of the vacuoles are smooth while those of the saccular cisternae are studded with ribosomes (plate 6, fig. 2; plate 7, fig. 1). The area occupied by the Golgi vacuoles appears hyaline in the lower power micrographs as well (plate 6, fig. 1) and can be easily distinguished from that occupied by the endoplasmic reticulum cisternae.

Plate 8

Figure 1. Chondrogenic tissue (chick, stage 31). A micrograph to make a general survey. The granular cisternae of the endoplasmic reticulum (er) are sometimes partly smooth and probably producing smooth vesicular profiles (arrows). In the Golgi apparatus some smooth vesicles (v) are apparently contributing to the formation of the Golgi lamellae (gl), and at the same time some lamellae are in the process of swelling up, probably to form the Golgi vacuoles (gv). The nuclear envelope (ne) can be seen in the process of 'blebbing' and the nucleolus has its characteristic particulate (pn) and fibriller (fn) areas. M1, X 20,700.

Figure 2. Chondrogenic tissue (chick, stage 31). Part of a chondroblast showing a transition between granular endoplasmic reticulum (below the arrow) and lamellar profile of the Golgi apparatus (above the arrow). Also note the adlineation of Golgi vacuoles (gv) probably to form large vacuoles; the structure of the cytosome (cs); and pinocytotic vesicle (p). M1, X 31,000.

Figure 3. Chondrogenic tissue (chick, stage 31). Notice several multivesicular bodies (mv), lipid droplet (l), and a pinocytotic vesicle (p), whose contents are similar to and continuous with the surface coat. Swelling of the Golgi lamellae (gl) can also be seen. M1, X 20,700.



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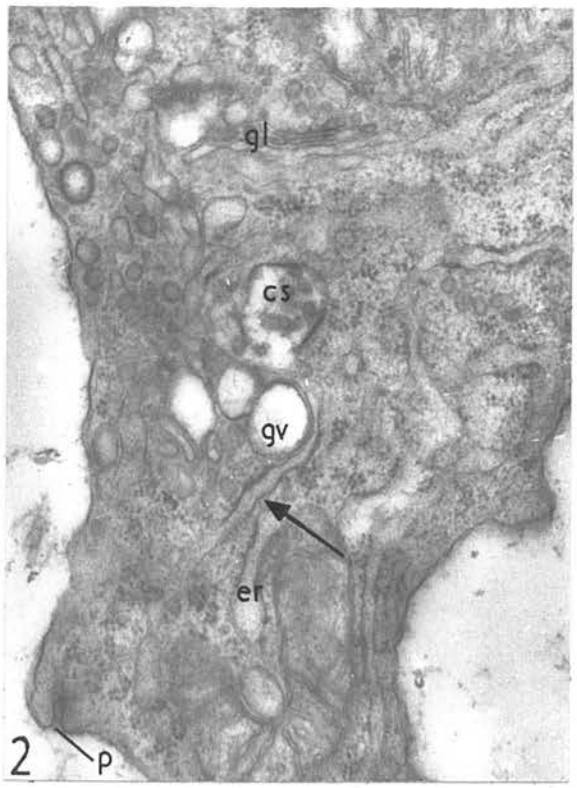

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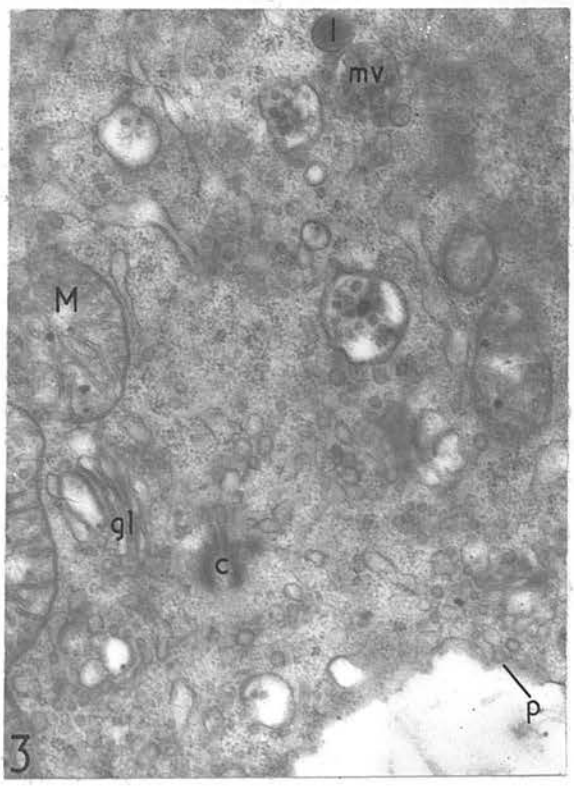

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Some of the cytosomes contain lipids while others have varying numbers of small rounded vesicles, about 50 to 70 mμ in diameter, of moderate electron density (multivesicular bodies) and still others may contain an amorphous ground substance in addition to the vesicles (plate 8, figs. 2 and 3).

Moreover, some vesicular invaginations of the plasmalemma, probably pinocytotic vesicles, are noticeable in this stage (plate 8, figs. 2 and 3; see also Glauert et al. 1969). The contents of the invaginations are sometimes continuous with and have a moderate electron density similar to the cell surface coat.

The 'ecdysis', 'excortication', or 'delamination' is another interesting feature of the chondroblasts. The electron microscopic evidence for it consists of frequent association between the cell plasmalemma and the fibrous elements of the extracellular phase (plate 6, figs. 2 and 3). The fibres do not penetrate deep in the cell cytoplasm; they are either continuous with the plasmalemma itself or seem to penetrate just under it. Moreover, the otherwise well-defined plasmalemma usually appears indistinct or discontinuous at such points of contact between plasmalemma and the fibres. A study of micrographs points to two types of ecdysis - one connected with the bursting of vacuoles (plate 6, fig. 2), and the other which is independent of it (plate 6, fig. 3).

The extracellular phase at this stage consists of an amorphous largely electron translucent ground substance with a fair amount of short unbanded fibres scattered throughout it and measuring 9-18 mμ in thickness. Rarely some electron dense granules are also observed in association with the fibres.

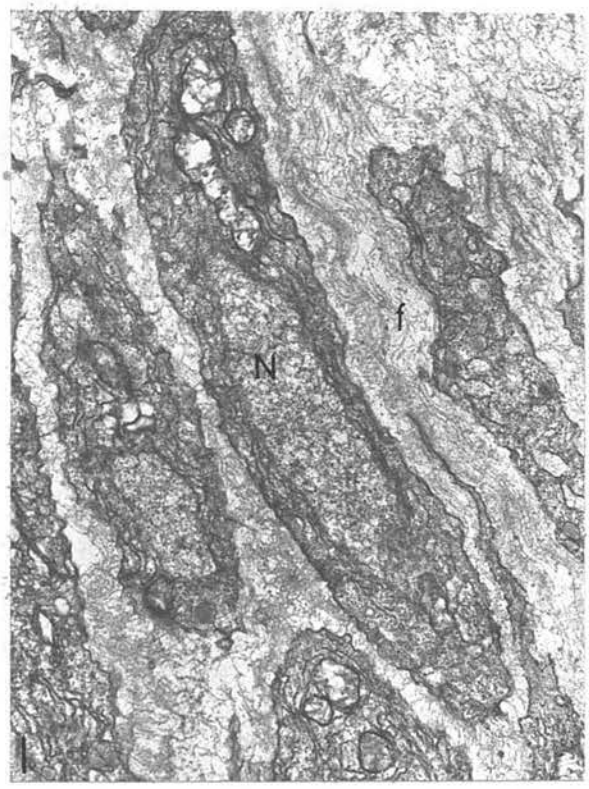
(d) Late chondrogenic tissue (chick, stage 37). The late chondroblasts differ from the early chondroblasts (stage 31) mainly in being more regular in shape and having still better developed Golgi apparatus, though the Golgi lamellae are fewer. The difference between the Golgi vacuole area and the endoplasmic

Plate 9

Figure 1. Late chondrogenic tissue (chick, stage 37). The thin and elongated cells of perichondrium have elongated nuclei (N). There is a large extracellular phase filled with fibrous material (f) but lacks any electron dense granule. The Golgi apparatus is not well developed. M1, X 13,700.

Figure 2. Late chondrogenic tissue (chick, stage 37). These cells from near the periphery show interlacing cisternal profiles of the endoplasmic reticulum (er) and the electron translucent area occupied by Golgi vacuoles (gv). Also note that extracellular phase contains electron dense granules. M1, X 27,800.

Figure 3. Late chondrogenic tissue (chick, stage 37). The micrograph shows parallel running cisternae of the endoplasmic reticulum (er) in the cortical area of one late chondroblast, and double-membraned cytosome (cs) and the Golgi vacuoles (gv) in another. M1, X 40,600.



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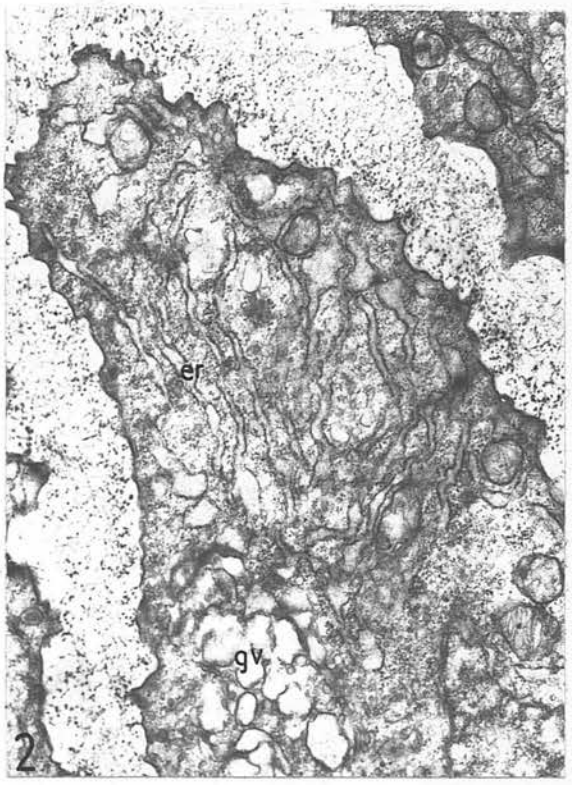

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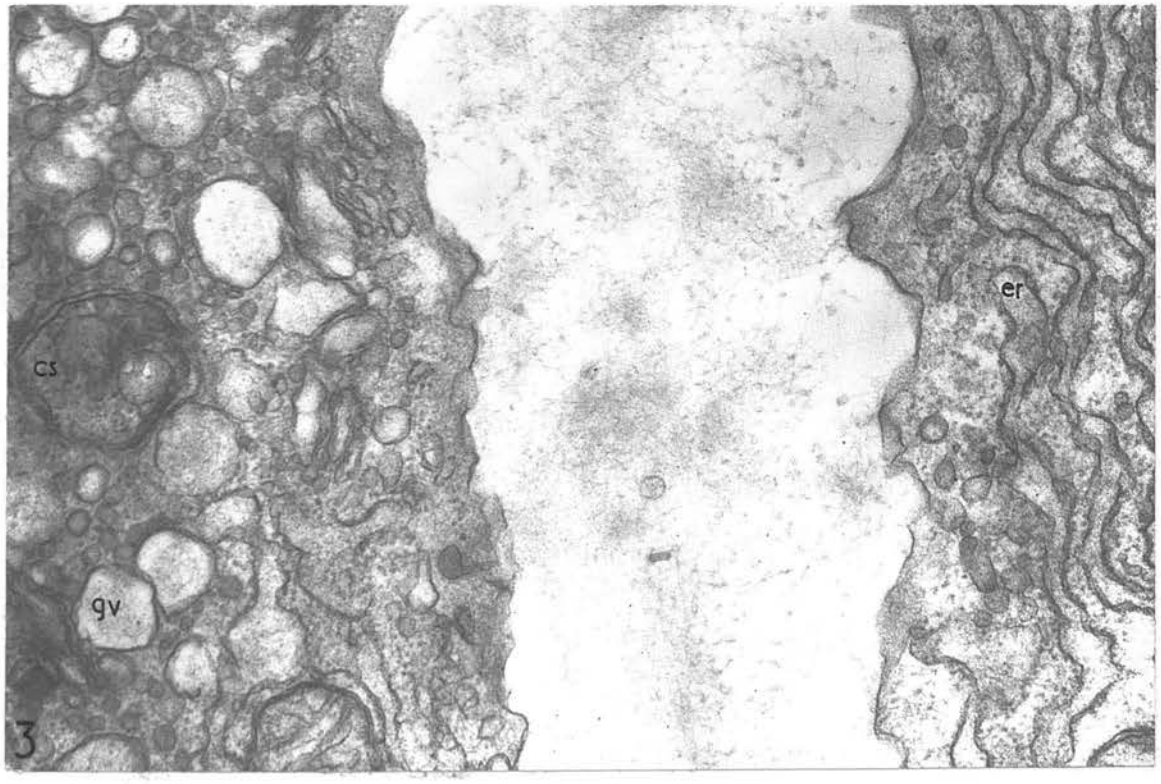

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reticulum area remains apparent (plate 9, fig. 2). The endoplasmic reticulum is extensive and very frequently dilated forming saccular cisternae that are up to 2.2 μ in diameter. The reticulum is usually in the form of parallel arrays of elongated cisternal profiles in the cortical region of the cell (plate 9, fig. 3) and in the form of an interlacing network in the central area (plate 10, fig. 1). The cytosomes are also well developed (plate 10, fig. 1) and are some times bounded by double membranes. The extracellular matrix is extensive and consists of an amorphous ground substance with fibres as well as electron dense granules.

The perichondrium consists of 4 to 5 layers of thin elongated cells and an extracellular phase which contains a densely packed fibrous matrix (plate 9, fig. 1). The perichondroblasts have an elongated nucleus and the vacuolar component of their Golgi apparatus is not as well developed as that of the chondroblasts.

(e) Epiphyseal embryonic cartilage (chick, stage 39).

(i) Chondrocytes The chondrocytes at lower magnification are quite regular in outline. They have almost no intercellular connections and are separated from each other by a very extensive extracellular phase. Moreover, all the cell organelles, as compared to those of the mesenchyme cells at stage 23, appear much more electron dense, a feature usually attributed to a lower water content of the tissue.

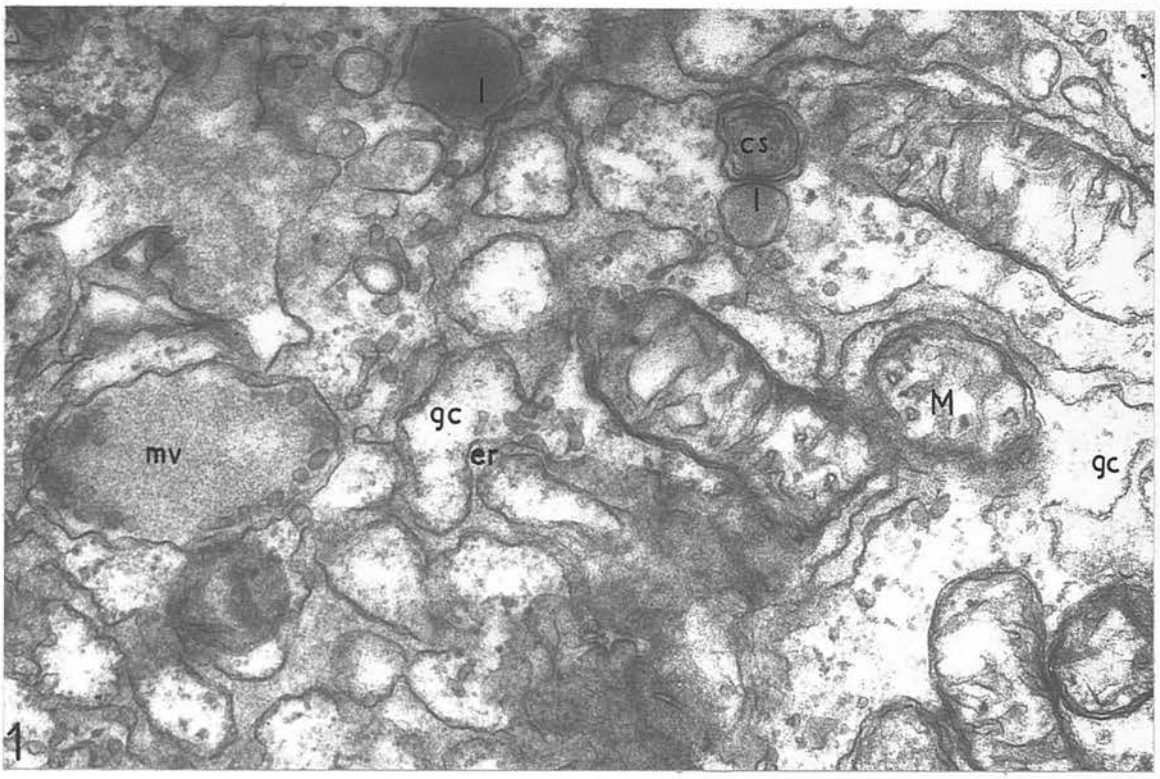
The nucleus is small and eccentrically placed (plate 10, fig. 3), and the perinuclear cisterna is often continuous with the endoplasmic reticular cisternae (plate 12, fig. 2). In sections of glutaraldehyde-osmium fixed tissue, the nuclear matrix consists of a ground substance interspersed with small lumps of electron dense chromatin which also forms a thin layer on the inner side of the nuclear envelope (plate 11, figs. 1; plate 12, fig. 2). On the other hand, the nuclear

Plate 10

Figure 1. Late chondrogenic tissue (chick, stage 37). This chondroblast shows an interlacing network of endoplasmic reticulum (er); multivesicular body (mv); lipid droplets (l) and other type of cytosome (cs). M1, X 40,600.

Figure 2. Epiphyseal embryonic cartilage (chick, stage 39). A low power electron micrograph showing an extensive extracellular phase (ep), consisting of fibres and granules, and completely independent chondrocytes. The matrix reaches right up to the chondrocytes, there being no pericellular lacunae. M2, X 9,000.

Figure 3. Epiphyseal embryonic cartilage (chick, stage 39). In this micrograph notice the eccentrically placed nucleus (N) and the difference between areas of the Golgi vacuoles (gv) and those of the saccular cisternae of the endoplasmic reticulum (er). M1, X 3,400.



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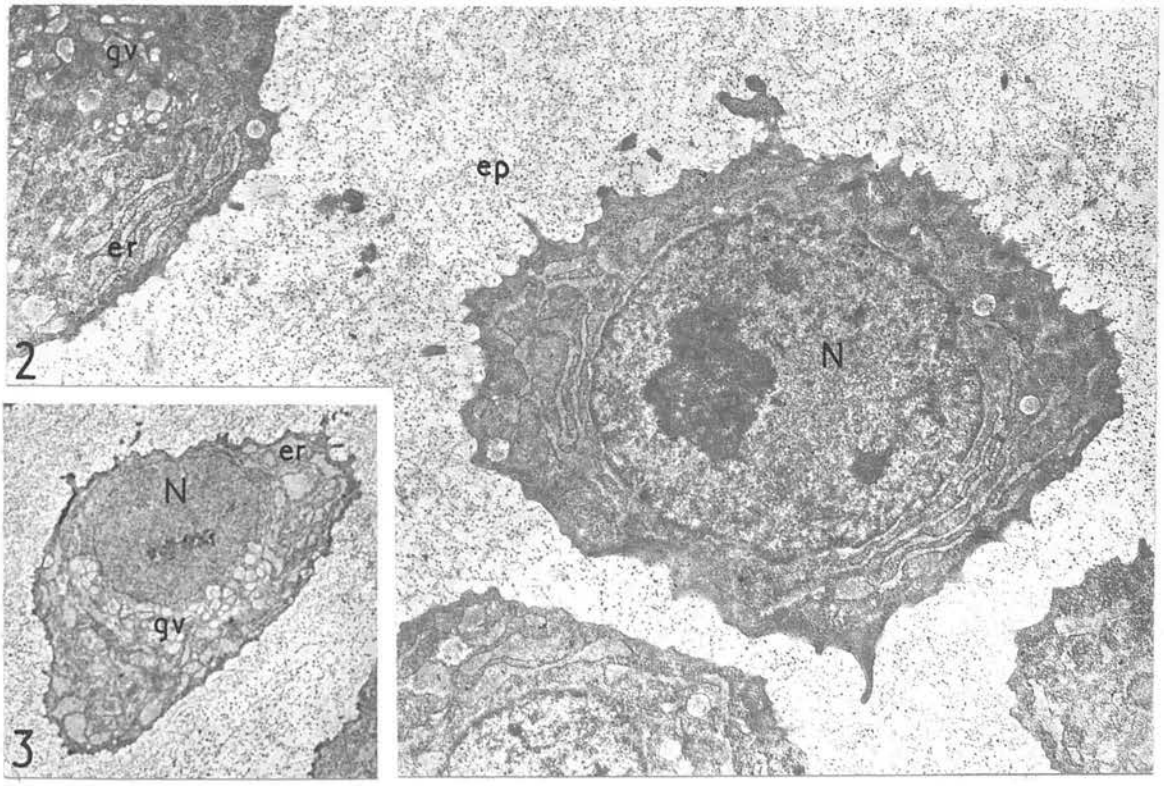

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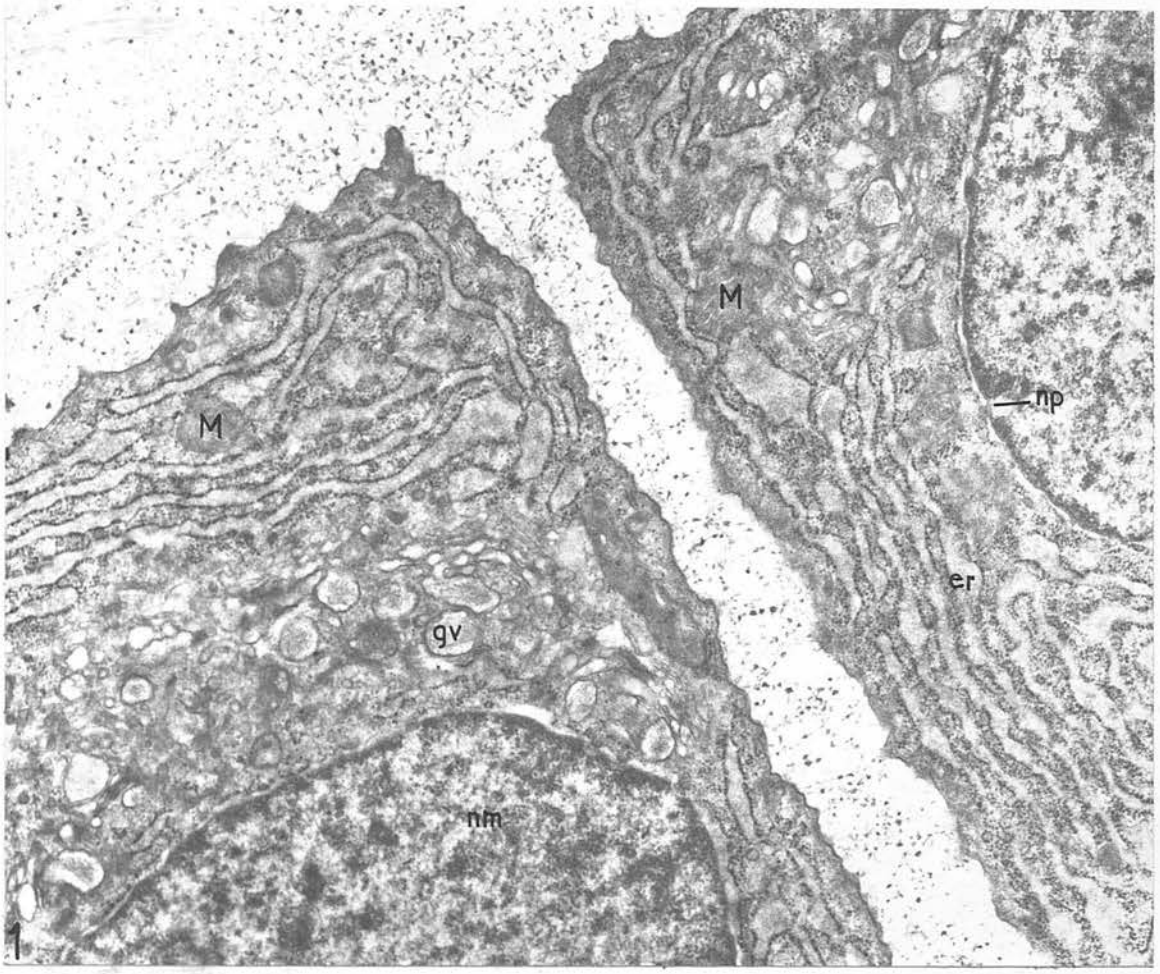
Plate 11

Figure 1. Epiphyseal embryonic cartilage (chick, stage 39). An electron micrograph to make a general survey. The chromatin, in addition to its distribution in the nuclear matrix (nm), forms a continuous layer adjacent to the inner side of nuclear envelope except at the nuclear pores (np); the outer nuclear membrane is studded with ribosomes; the endoplasmic reticulum (er) has parallel arrays of elongated cisternae; the mitochondria (M) have dense matrix and well preserved cristae; the Golgi vacuoles (gv) contain chondrogen granule. M2, X 20,700.

Figure 2. Epiphyseal embryonic cartilage (chick, stage 39). Part of a chondrocyte showing a Golgi vacuole (gv) whose membrane is fused with plasmalemma, and which contains a chondrogen granule (cg). Compare the structures in the chondrogen granule to the corresponding structures in the extracellular phase (ep), and to the amorphous, homogeneous and electron dense contents of the saccular cisternae of the endoplasmic reticulum (er). M1, X 25,700.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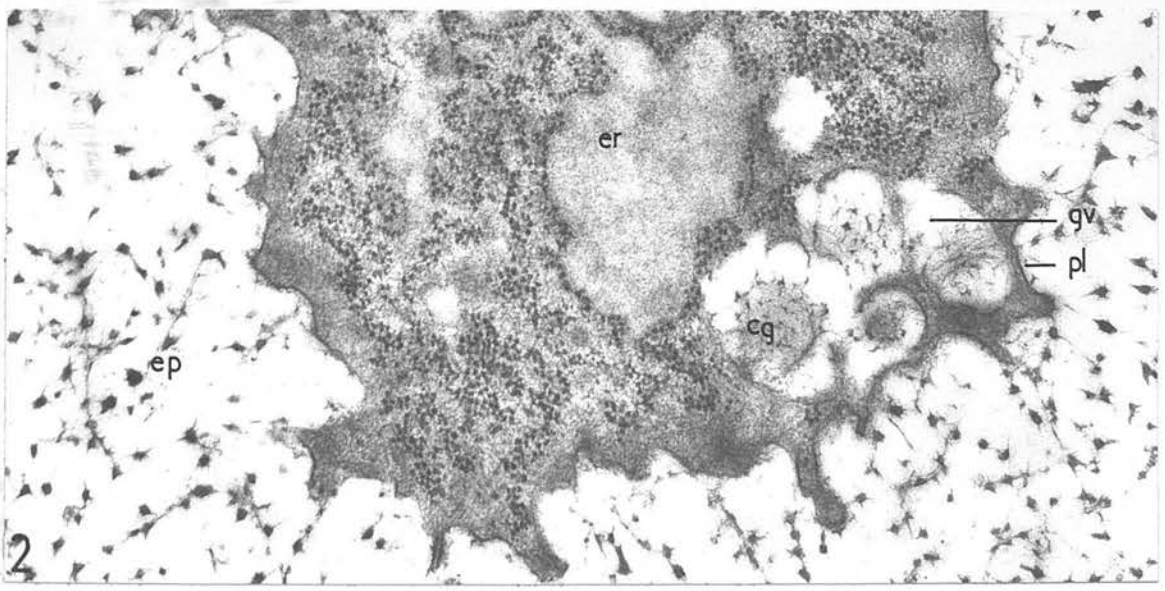
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matrix from tissue fixed in osmium tetroxide alone, consists of a rather homogeneous mass lacking any electron dense chromatin (plate 12, fig. 1). In the nucleolus, the fibrillar areas tend to lie near the periphery of the organelle.

The moderate electron density of the ground cytoplasm is accentuated by the presence of numerous highly electron dense polysomal groups which usually appear in helical or circular patterns (plate 12, fig. 2).

The endoplasmic reticulum remains very well developed and its membranes are still rough and the cisternal contents are moderately electron dense and amorphous (plate 11, fig. 2). The saccular cisternae of the endoplasmic reticulum are numerous and are large, measuring up to 5.3 μ in diameter. The cisternae are not seen opening to the outside though they frequently reach close to the plasmalemma. There are very few vesicular profiles of the endoplasmic reticulum.

The well developed juxtanuclear Golgi apparatus differs from that of the chondroblasts chiefly in the relative abundance of the Golgi vacuoles and the scarcity of the lamellae and vesicles (plate 12, fig. 1). Moreover, the vacuoles, as well as the slightly larger vesicles, almost invariably contain a chondrogen granule. This granule, under high magnification, appears remarkably similar to some components of the extracellular phase (plate 11, fig. 2). It consists of a moderately electron dense amorphous substance having small electron opaque granules with attached small fibrillar elements. However, both the small granules and the fibrils in the vacuoles are respectively smaller and thinner compared with the corresponding components of the extracellular phase. The Golgi vacuoles closely approach the plasmalemma and sometimes their membrances fuse with it (plate 11, fig. 2).

The differences between the Golgi vacuoles and the saccular cisternae of the endoplasmic reticulum, as noted in the chondroblasts, still persist and the areas occupied by these two organelles can be easily distinguished in the low power electron micrographs (plate 10, figs. 2 and 3).

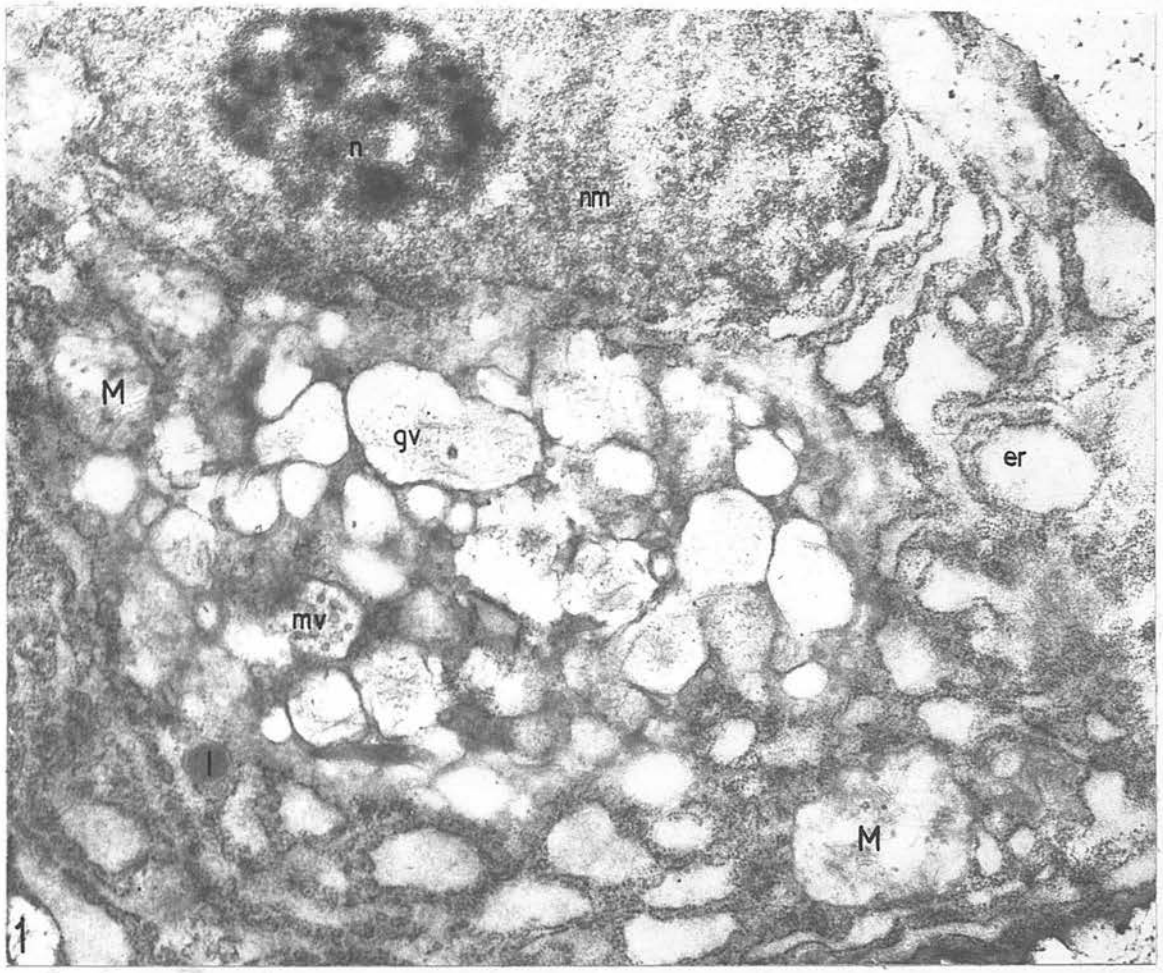
Plate 12

Figure 1. Epiphyseal embryonic cartilage (chick, stage 39). An electron micrograph from osmium tetroxide fixed tissue to show the poor preservation of chromatin in nuclear matrix (nm), nuclear envelope, and mitochondria (M). Note the abundance of the Golgi vacuoles (gv) and also a multivesicular body (mv) and a lipid droplet (l). M1, X 20,700.

Figure 2. Epiphyseal embryonic cartilage (chick, stage 39). Notice the continuity between the cisternae of the endoplasmic reticulum and the perinuclear cisterna (arrow), and also ribosome studded outer nuclear membrane and the distribution of chromatin. M2, X 20,700.

Figure 3. Epiphyseal embryonic cartilage (chick, stage 39). Part of a chondrocyte showing granular endoplasmic reticulum with polysomal groups, arranged in helical and circular patterns, attached to them (arrows). Also note the poorly preserved mitochondria (M). M1, X 31,000.

Figure 4. Epiphyseal embryonic cartilage (chick, stage 39). The extracellular phase showing electron opaque granules (g) and fibres (f); some of the latter show a faint periodicity. M1, X 91,000.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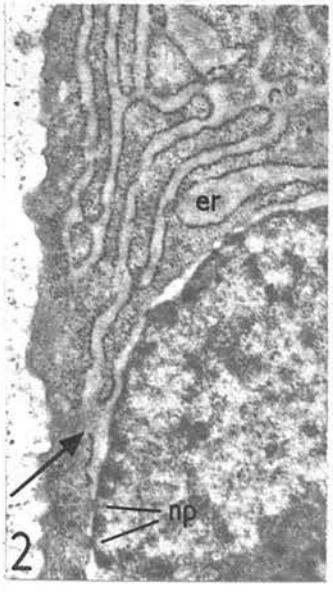
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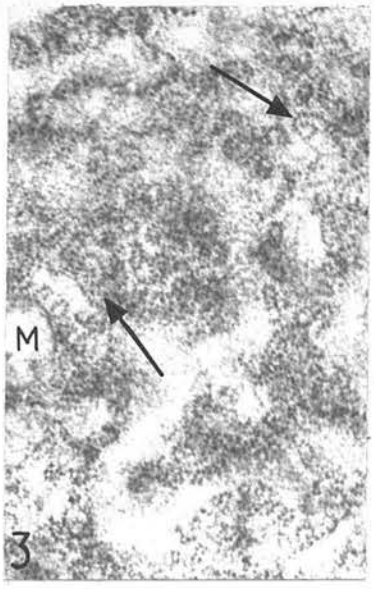
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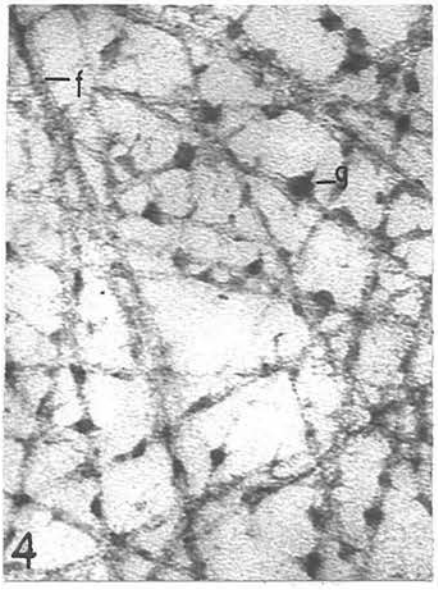
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Mitochondria, like the nuclei , give a different appearance, depending on the fixative used. In the osmium tetroxide fixed tissue they seem rather extracted and have an almost electron transparent matrix, few small cristae and a few granules (plate 12, figs. 1 and 3). In glutaraldehyde-osmium fixed tissue, on the other hand, the matrix is moderately electron dense and the cristae well preserved (plate 11, fig. 1).

The extracellular phase consists of an amorphous electron translucent ground substance and a large amount of fibrous material (plate 12, fig. 4). The 20 to 25 mμ thick fibres are usually long and straight, each consists of 2 or 3 thin fibrils, 7 - 10 mμ in thickness. These fibrils sometimes show a periodicity of 9 mμ with a light band of 3 mμ and a dark band of 6 mμ (plate 12, fig. 4). Along the course of fibre, polygonal electron opaque granules are distributed at an interval of approximately 30 mμ. The granules also lie free in the interfibrous area and usually measure 20 - 30 mμ, but may be as large as 45 mμ. Though no ecdysis is noticeable at this stage, the fibres often are in contact with the plasmalemma and sometimes even appear to enter the cytoplasm.

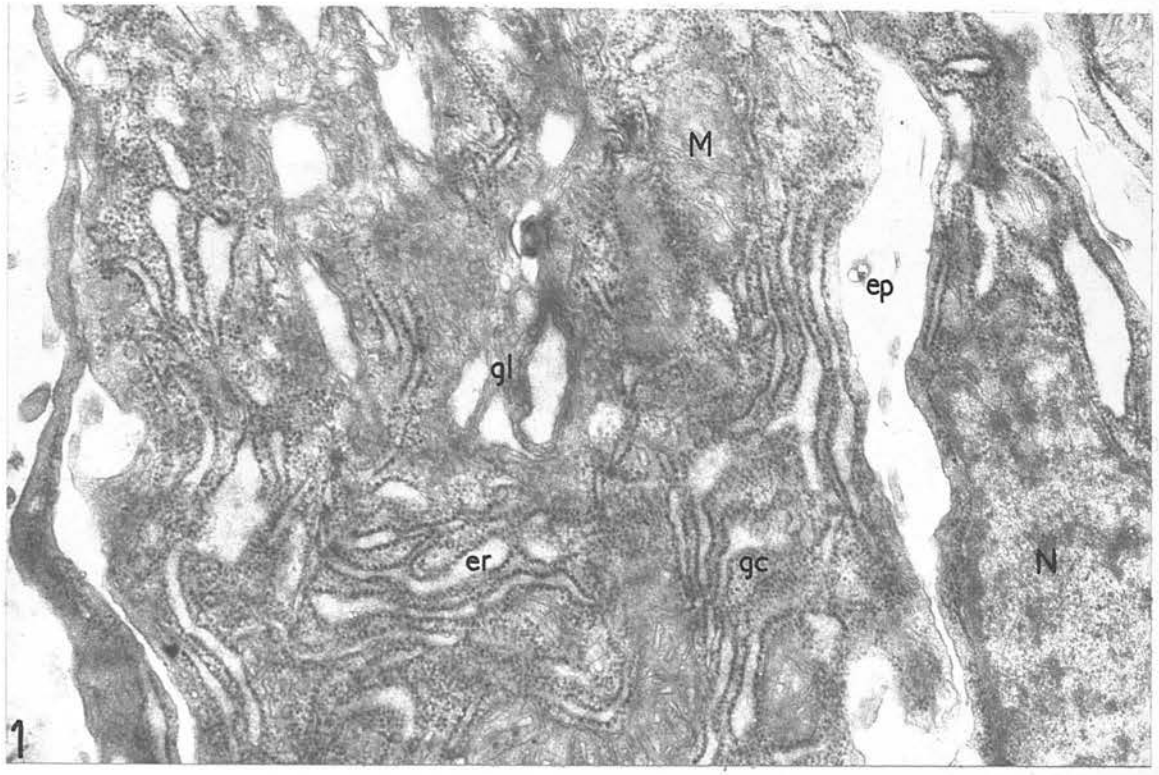
(ii) Perichondrium The perichondrial cells differ from the chondrocytes both in their general morphology as well as in ultrastructure. In sections, they appear very flattened and elongated. These cells occur as two types, one which is regarded as the normal perichondrial cells and the second called hypertrophied cells.

In the cytoplasm of normal perichondrial cells the ground substance is amorphous, homogeneous and moderately electron dense. It contains numerous ribosomes. The endoplasmic reticulum consists almost exclusively of granular elongated cisternae, which run parallel to each other, are often convoluted and only infrequently dilated to form saccular cisternae (plate 13, fig. 1).

Plate 13

Figure 1. Epiphyseal embryonic cartilage (chick, stage 39). The normal perichondrial cell with a moderately electron dense ground cytoplasm (gc), elongated cisternae of the endoplasmic reticulum (er), and convoluted lamellae of the Golgi apparatus (gl). There are almost no Golgi vacuoles. The mitochondria (M) are very well preserved. M2, X 25,700.

Figure 2. Epiphyseal embryonic cartilage (chick, stage 39). The cell in top left hand corner is a normal perichondrial cell, the one below it is in the process of hypertrophy and one at the bottom is well advanced in hypertrophy. Notice the gradual change in the electron density of the ground cytoplasm (gc), disappearance of free polysomes, and thinning of plasmalemma (pl), M2, X 20,700.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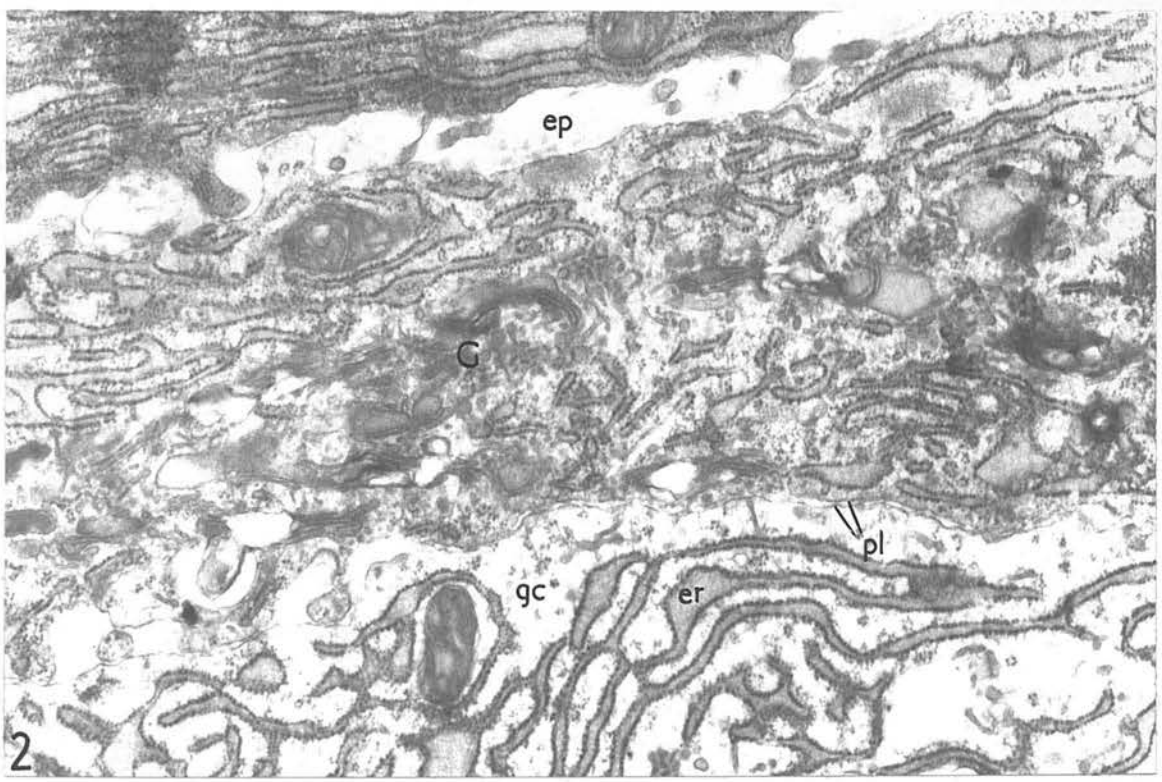
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Their contents are amorphous, moderately electron dense and often continuous with those of the perinuclear cisterna. The Golgi apparatus consists of a number of thin lamellae, usually arranged in stacks of 3 to 6, and a few vesicles but no vacuoles. The lamellae, around 20 mμ in diameter, are sometimes long and convoluted. The vesicles are around 35 to 70 mμ in diameter.

The extracellular phase contains indistinctly banded fibres, which do not have any electron dense granules associated with them.

In the study of cells undergoing hypertrophy and probable degeneration the most noticeable change during this process occurs in the ground cytoplasm, which is moderately electron dense in a normal cell but almost electron transparent in a hypertrophied cell (plate 13 fig. 2). Associated with and contributing to it is the disappearance of free polysomes; however, the reticulum-attached polysomes persist in the hypertrophied cells. The decrease in the density of the ground cytoplasm gives an illusion of an increase in electron density of the contents of the endoplasmic reticulum cisternae. It may be noted that the lysosomes or cytosomes are almost always absent in the cells undergoing hypertrophy. The direct causative factor responsible for the degenerative changes remains obscure.

2. DIFFERENTIATION OF CHICK LIMB-BUD CARTILAGE IN TISSUE CULTURE

I. Light microscopic observations

The cartilage nodules, developed in the tissue culture as early as 40 hours, are usually spheroidal structures. The general arrangement of cells inside the nodules, at the light microscopic level, is concentric and comparable to that of the chondroblasts differentiating in vivo (see page 32). The cells in the centre, like the typical chondroblasts, have a scalloped outline, but may sometimes be rounded (plate 14, fig. 2). Towards the periphery of the nodule the cells are usually thin, elongated, closely-packed and concentrically arranged,

Plate 14

Figure 1. Cartilage of chick differentiated in tissue culture. Notice the concentric arrangement of cells on the periphery of cartilage nodule and a fair amount of the extracellular phase towards the centre of nodule. Compare it to plate 2, figure 1, in which cartilage condensation differentiated in vivo is seen. M1, X 250.

Figure 2. Cartilage of chick differentiated in tissue culture. Compare it to figure 1: note the rounded shape of the centrally located cells and an extensive extracellular phase towards the centre of nodule. M1, X 375.

Figure 3. Cartilage of chick differentiated in tissue culture. Note the eccentric nuclei (N) and a small amount of fibrillar matrix in the extracellular phase (ep). M1, X 4,400.

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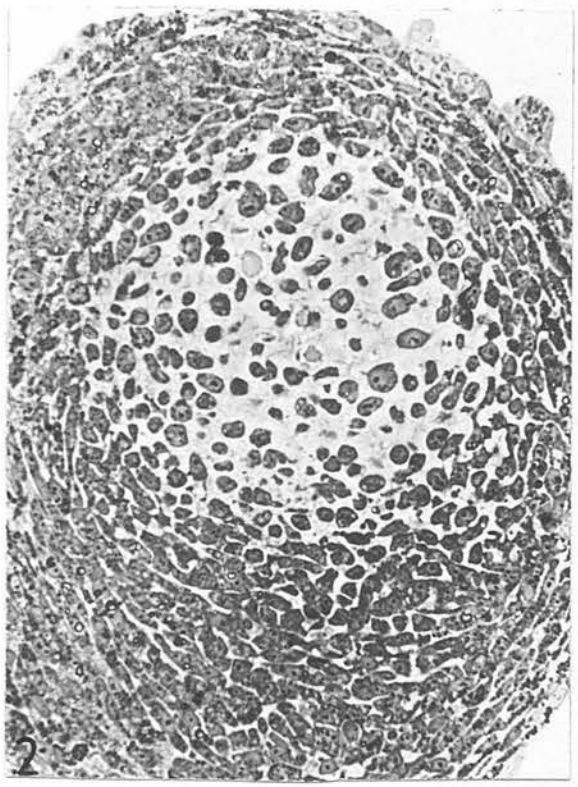

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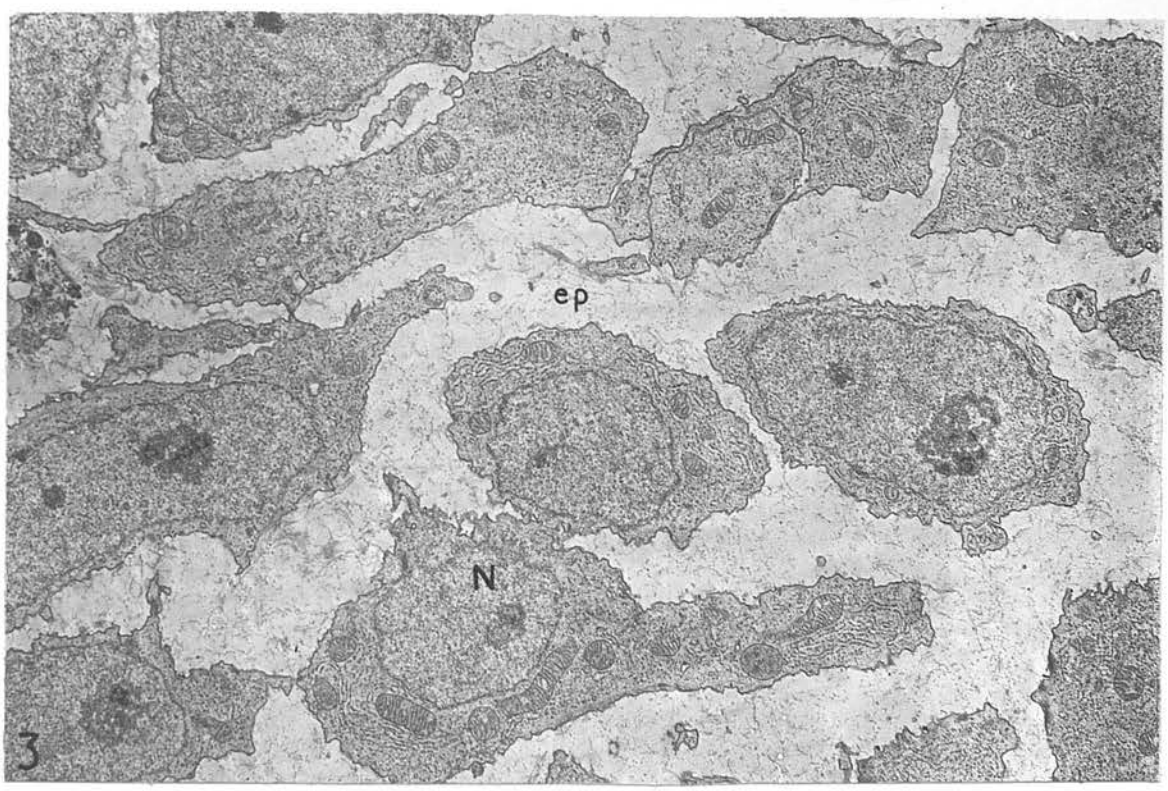

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(plate 14, figs. 1 and 2). These cells could be designated as the perichondrial cells. The extracellular phase is sometimes extensive, particularly in the centre of the nodules (plate 14, fig. 2), and stains metachromatically with toluidine blue. The chondroblasts in the process of mitotic division are also noticeable in some cases.

II. Electron microscopic observations

The chondroblasts differentiated in tissue culture are noticeably different from those differentiated in vivo, particularly in the possession of some cytofibrils and cytosomes.

The nucleus is to some extent eccentrically placed (plate 14, fig. 3) and is enclosed in a 25 - 70 mμ thick nuclear envelope which is frequently studded with the ribosomes on its outer surface. The nuclear pores, around 70 mμ in diameter, are common (plate 15, fig. 1). The nuclear matrix is largely homogeneous and consists of granules and fibrils. Some chromatin material is also present and a part of it forms a very thin layer adjacent to the inner nuclear membrane. Usually there is only one nucleolus with morphology similar to the nucleolus of the chondroblasts differentiated in vivo; the more electron dense fibrillar areas of it are completely enclosed in less electron dense particulate areas.

In the cytoplasm the ribosomes are distributed either singly or as polysomes. The endoplasmic reticulum is granular and consists mainly of elongated cisternal profiles around 120 mμ in diameter. The dilated saccular cisternae are infrequent and measure up to 1.1 x 0.9 μ. The vesicular profiles of the endoplasmic reticulum are seldom seen (plate 15, fig. 3). The contents of the reticular system are homogeneous, amorphous and moderately electron dense. The Golgi apparatus, like that of the chondroblasts differentiated in vivo, consists of a very few lamellae, some vacuoles and many vesicles (plate 16, fig. 2).

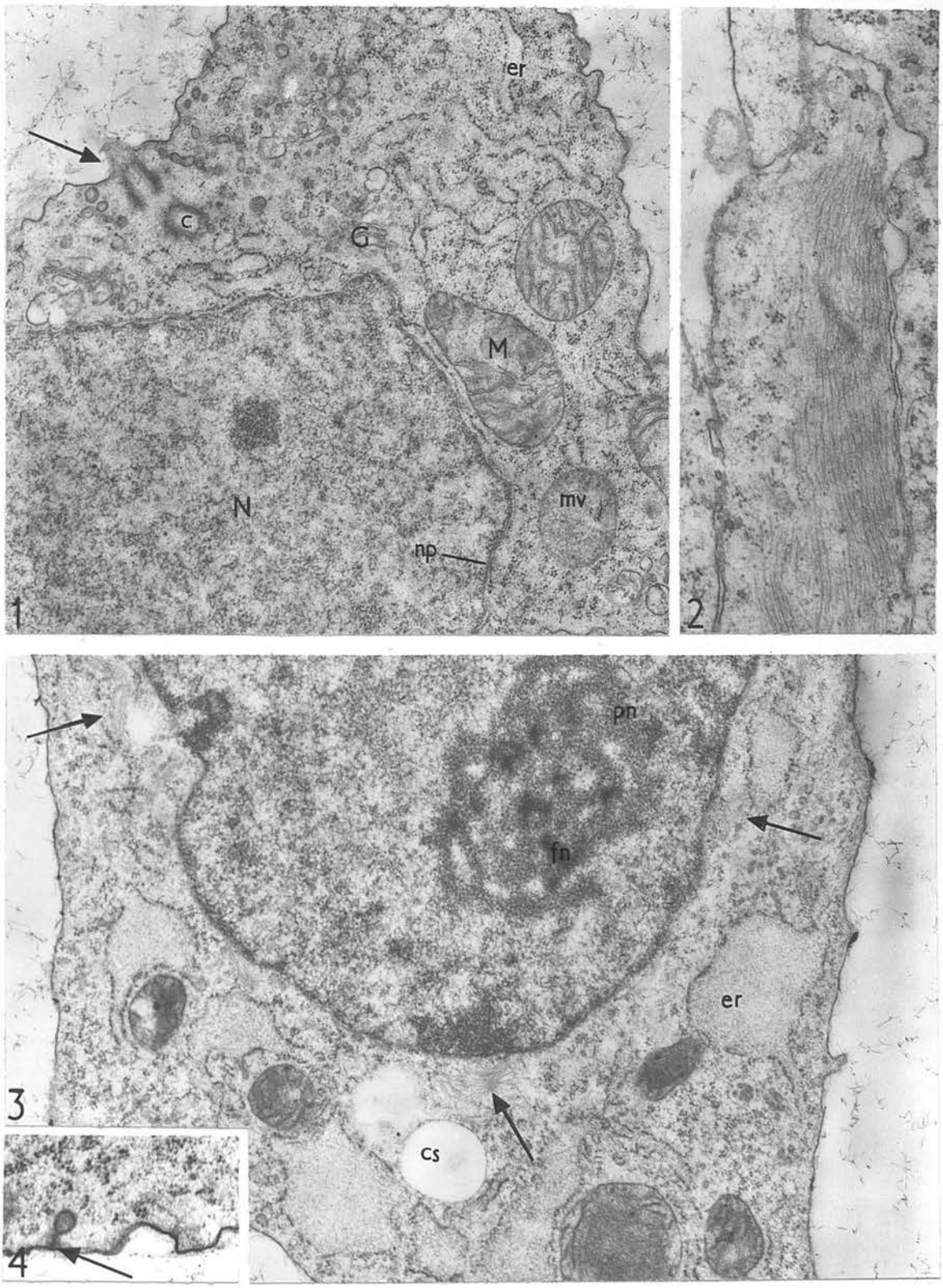
Plate 15

Figure 1. Cartilage of chick differentiated in tissue culture. A general survey of chondroblast showing mitochondria (M), Golgi apparatus (G), endoplasmic reticulum (er), multivesicular body (mv) and also a centriole (c) and cilium (arrow). The nuclear pores (np) can be seen in the nuclear envelope. M1, X 20,700.

Figure 2. Cartilage of chick differentiated in tissue culture. One of the chondroblast has its cytoplasm largely occupied by cytofibrils; such chondroblasts are found near the periphery of the nodule. M1, X 20,700.

Figure 3. Cartilage of chick differentiated in tissue culture. This chondroblast shows saccular cisternae of the endoplasmic reticulum (er), perinuclear cytofibrils (arrows) and a characteristic nucleolus with fibrillar regions (fn) enclosed in particulate regions (pn). M1, X 20,700.

Figure 4. Cartilage of chick differentiated in tissue culture. The micrograph shows a pinocytotic vacuole (arrow). M1, X 31,000.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The vacuoles may be as large as 0.7 μ in diameter and are usually electron translucent but sometimes contain a small quantity of materials which resemble certain elements of the extracellular phase. It appears as if the larger Golgi vacuoles are formed by the fusion of smaller ones. The area of the Golgi vacuoles and the saccular cisternae of the endoplasmic reticulum can be easily distinguished from each other, since, as in the chondroblasts differentiated in vivo, the area occupied by the Golgi vacuoles is electron translucent in appearance. Mitochondria are frequent and vary in shape and size from oval structures, about 0.5 μ in diameter, to elongated ones about 3 x 0.7 μ in size. They have a moderately electron dense matrix with a few mitochondrial granules and many mitochondrial cristae. Certain vesicles located below the plasmalemma, probably of pinocytotic origin, are of common occurrence (plate 15, fig. 4). A centriole as well as a cilium can be seen in plate 15, figure 1, though the cilia are uncommon.

Sometimes groups of fibrillar structures, the cytofibrils, around 5 - 8 mμ thick, can be seen in the perinuclear cytoplasm (plate 15, fig. 3). Moreover, in a few cases even bigger groups of slightly thicker fibrils, around 12 - 16 mμ thick, fill a considerable part of the cytoplasm of the cells situated on the periphery of the condensation (plate 15, fig. 2). The nature and frequency of both of these kinds of cytofibril, which were found only in tissue culture material, is unknown.

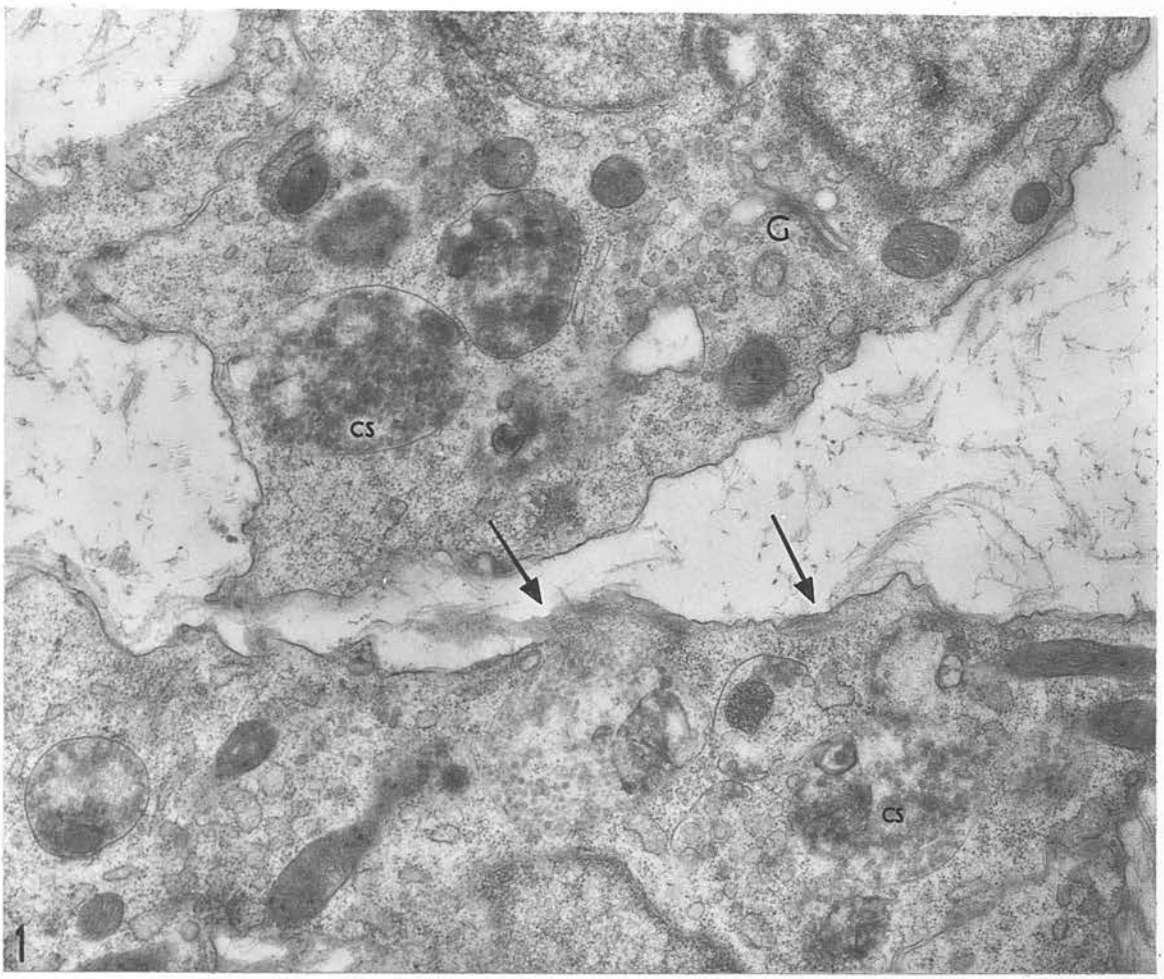
A variety of cytosomes of varying sizes and contents are frequently present (plate 16, figs. 1 - 3). They are usually around 0.5 to 0.8 μ in diameter although occasionally they may be as large as 1.6 x 2.5 μ. A large number of them contain small vesicles (50 - 70 mμ in diameter); some, in addition, contain an amorphous moderately electron dense mass and various ill-formed membraneous structures, while a few are most probably lipid droplets. Most of these structures are bounded by a single membrane, around 14 mμ thick, but some are

Plate 16

Figure 1. Cartilage of chick differentiated in tissue culture. The micrograph shows the relationship between extracellular fibres and apparently intracellular fibres (arrows). It also shows numerous cytosomes (cs) with heterogeneous contents and Golgi apparatus (G). M1, X 20,700.

Figure 2. Cartilage of chick differentiated in tissue culture. Notice the Golgi apparatus (G) and nearby it a cytosome (cs) with incomplete boundary. Also see one of the multivesicular bodies opening to the outside (arrow) and some other small vesicles outside the cell (arrow). M1, X 20,700.

Figure 3. Cartilage of chick differentiated in tissue culture. Three cytosomes of differing sizes are seen. Note the heterogenity of the contents of the largest one. M1, X 25,700.



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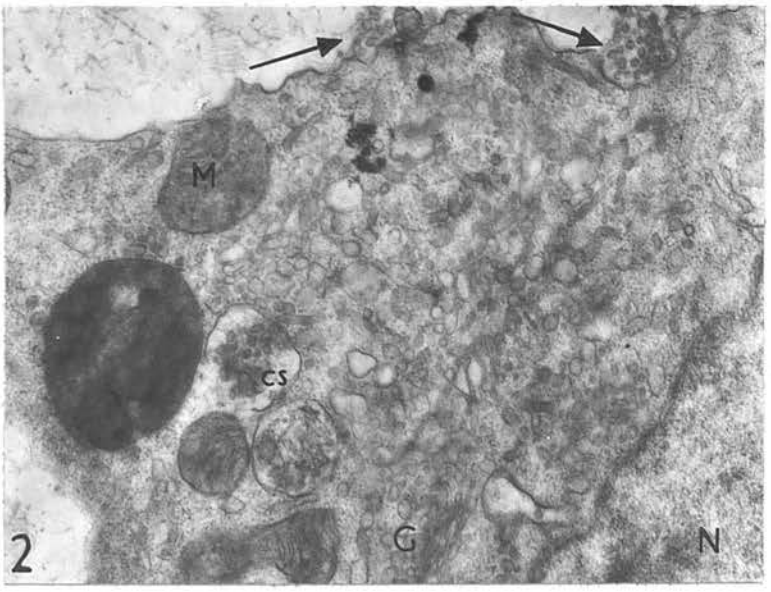

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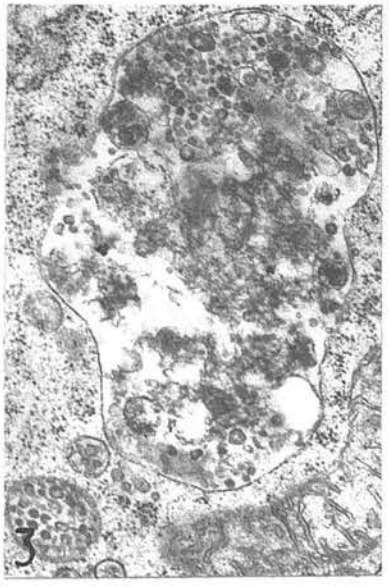

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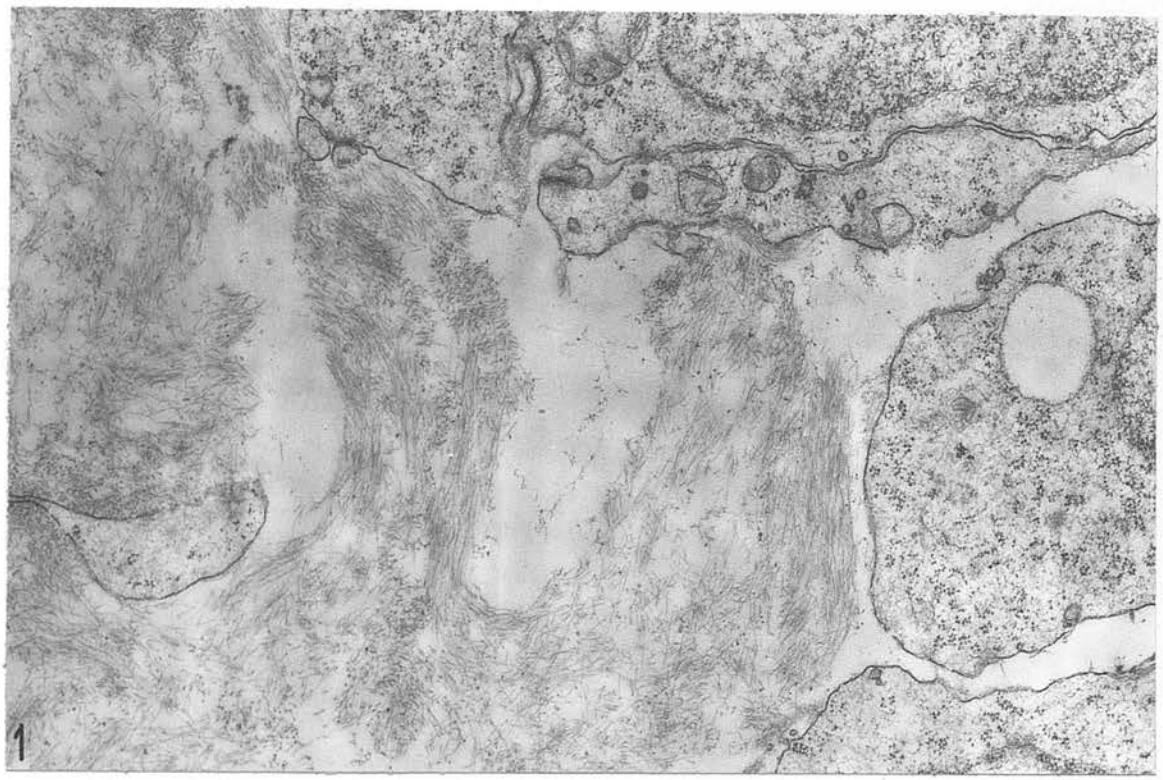
delimited by double membranes. Vesicles around 50 - 70 mμ in diameter and similar to those in the cytosomes are also present free in the cytoplasm, specially in the area of Golgi apparatus, suggesting the origin of cytosomes from this organelle. Moreover, the presence of cytosomes with incomplete boundary membranes in the Golgi area also indicates their Golgi origin, though the variation in their structure suggests that they may be formed in more than one way. In one case it is noted that the cytysome is opening to the extracellular phase (plate 16, fig. 2). A consideration of environmental conditions and various ultrastructural features of the chondroblasts in culture leads one to believe that at least some of the cytosomes are of lysosomal nature.

The process of ecdysis is more frequently found in these cells as compared to those differentiating in vivo. The fibres of the extracellular phase are often very near to or confluent with the plasmalemma (plate 17, fig. 2), and sometimes enter in the cortical cytoplasm, and run a short distance subjacent to the plasmalemma before merging into the cytoplasm (plate 16, fig. 1). At such points of contact, however, the plasmalemma seems discontinuous or indistinct, possibly because of tangential sectioning.

The extracellular phase contains numerous fibres embedded in a homogeneous amorphous mass (plate 17, fig. 1). The fibres, usually in bundles, are about 15 mμ thick, short and straight (plate 17, fig. 3). The presence of a relatively large number of fibres towards the periphery, rather than the centre, of the condensation is noticeable. The electron dense granules of the extracellular phase are neither as frequent nor as well developed as in the cartilage differentiated in vivo; and are usually completely missing in the cartilage from younger cultures.



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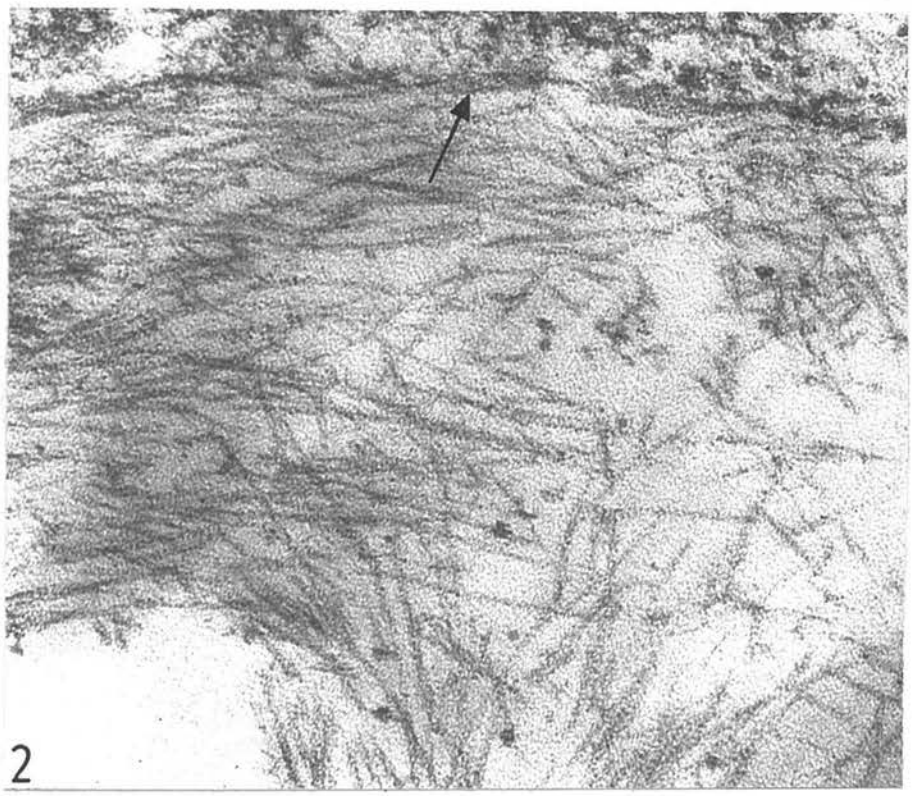

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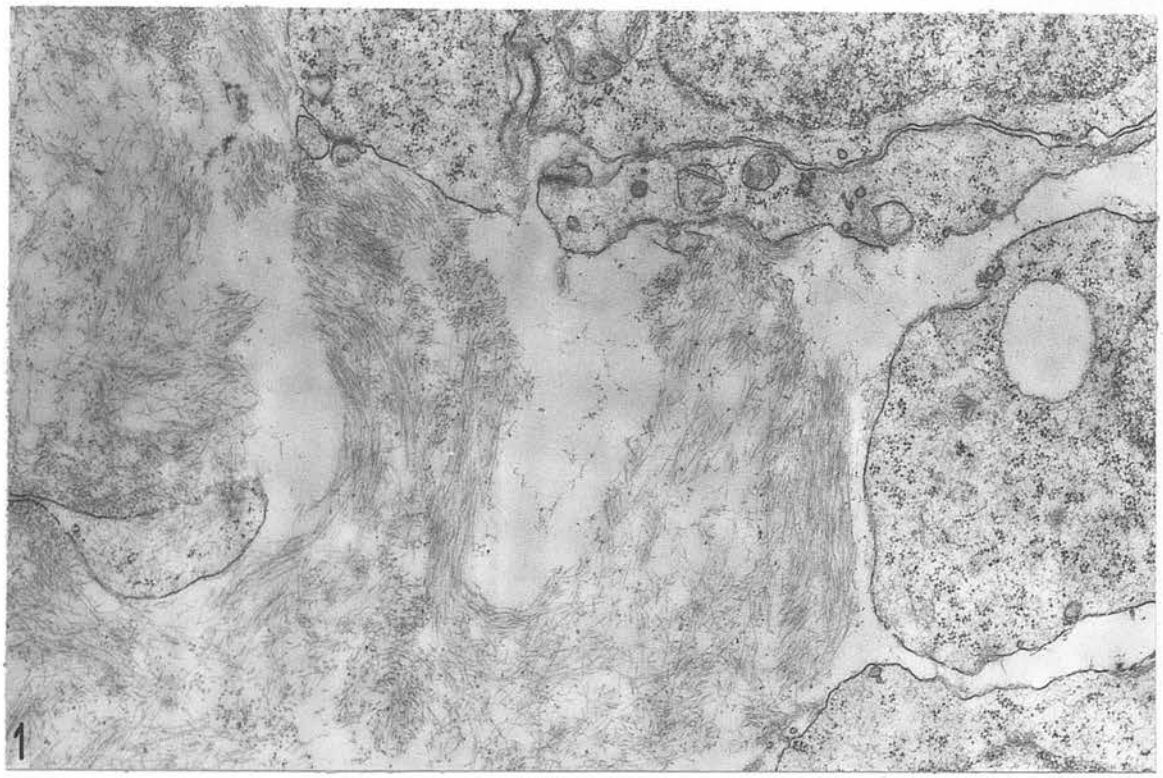

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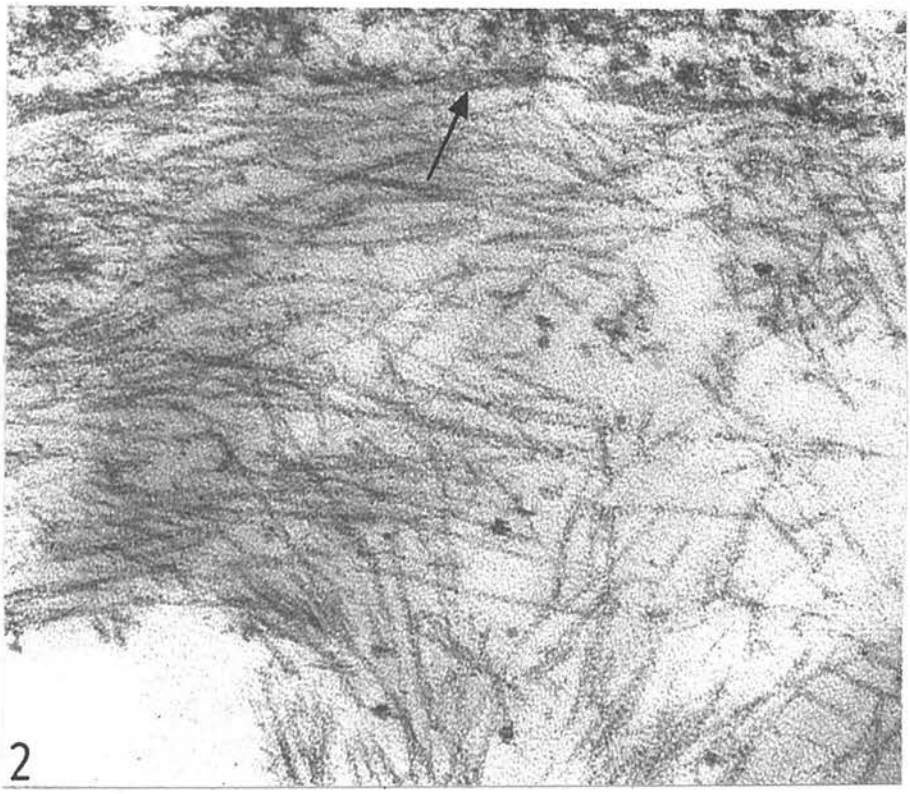

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3. DIFFERENTIATION OF HIND LIMB-BUD CARTILAGE IN MOUSE IN VIVO

I. Light microscopic observations

(a) Mesenchyme (mouse, 12-day-old embryo). The mesenchymal tissue of the mouse, as compared to that of the chick, consists of closely-packed, rounded cells (plate 18, fig. 1). The cells have a very large, centrally placed nucleus with one, sometimes two or rarely three, darkly staining nucleoli. The cytoplasm is present as a thin layer around the nucleus: nucleocytoplasmic ratio is around 4. The cell boundaries can usually be followed in the sections and, in contrast with the chick mesenchyme, there are very few cytoplasmic extensions. The extracellular phase is hyaline and inextensive. Cells undergoing mitotic division are frequently encountered.

(b) Prechondrogenic tissue (mouse, 13-day-old embryo). At this stage the onset of cartilage condensation can be seen at the light microscopic level (plate 18, fig. 2). In the centre of the condensation the prechondrogenic cells do not differ from the mesenchyme cells. The cells located at the periphery of condensation give an impression of being concentrically arranged, but they are not yet morphologically distinguishable from the more centrally placed cells.

(c) Early chondrogenic tissue (mouse, 16 days old embryo). The condensation is now distinctly recognisable, and the cells may be termed chondroblasts (plate 18, fig. 3). Their nuclei are still very large though eccentrically placed; the nucleocytoplasmic ratio is about 2. The individual chondroblasts are almost always completely separated from each other. There is a large extracellular phase but it contains very little of fibrous matrix. The peripheral cells as compared to the chondroblasts, are smaller, more closely packed and concentrically arranged. However, as contrasted to the chick perichondroblasts, they are neither thin nor elongated in sections.

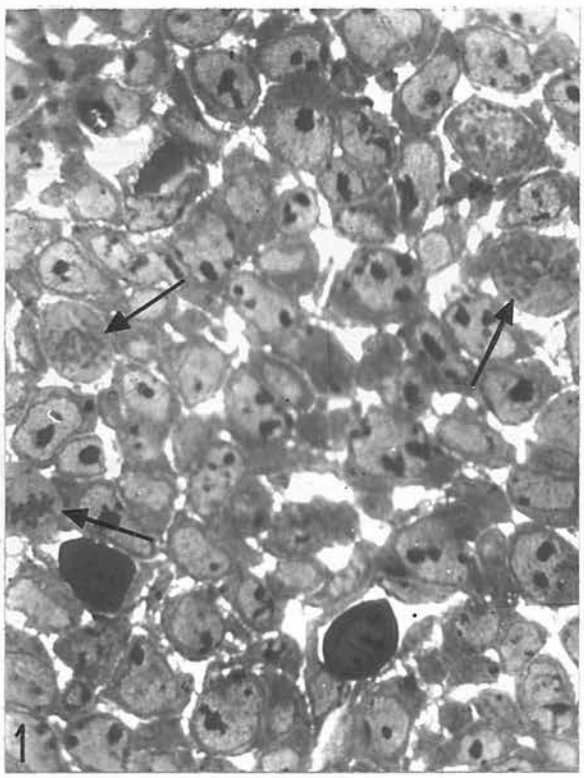
Plate 18

Figure 1. Mesenchyme (mouse, 12-day-old embryo). These cells as compared to similar cells in the chick (plate 1, figure 1) are rounded, more closely packed and with fewer cytoplasmic extensions. The nucleocytoplasmic ratio is high and mitoses (arrows) are frequent. M1, X 1,100.

Figure 2. Prechondrogenic tissue (mouse, 13-day-old embryo). The area of future cartilage can be just detected, since the cells on the periphery of condensation are somewhat concentrically arranged. M1, X 1,100.

Figure 3. Early chondrogenic tissue (mouse, 16-day-old embryo). The condensation is well marked and the early chondroblasts have eccentric, though large, nuclei. The extracellular phase is not yet extensive. The cells on the periphery of the condensation, as compared to similar cells in the chick, are neither thin nor elongated. M1, X 1,100.

Figure 4. Late chondrogenic tissue (mouse, 18-day-old embryo). A characteristic appearance of mouse epiphyseal cartilage can be seen. Glycogen-laden chondroblasts with small eccentric nuclei are interspersed in an extensive matrix. The glycogen can be seen even in a mitotic cell (arrow). This tissue differs from corresponding tissue in chick (plate 2, figure 3) not only in the presence of glycogen but also in shape and size of the perichondrial cells. M4, X 1,100.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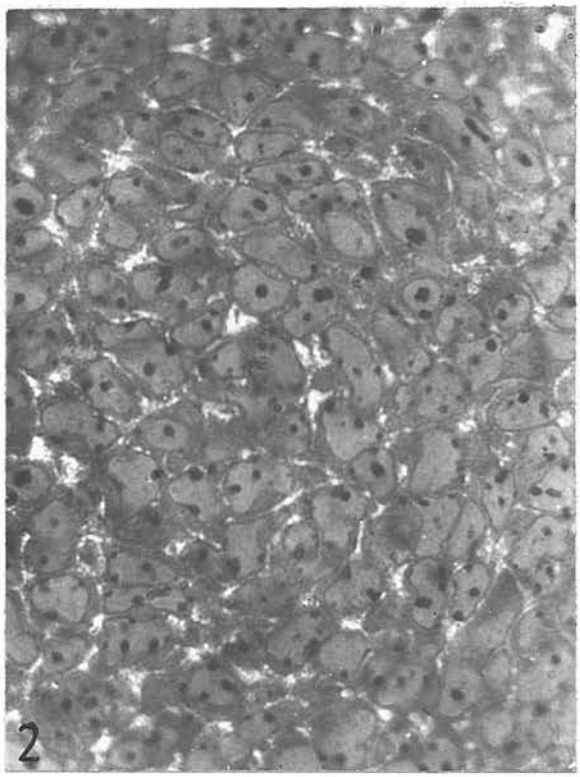
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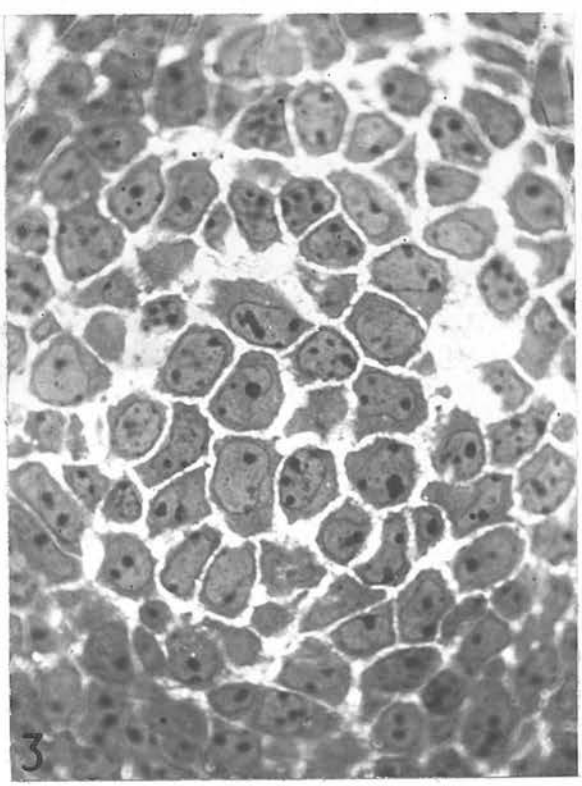
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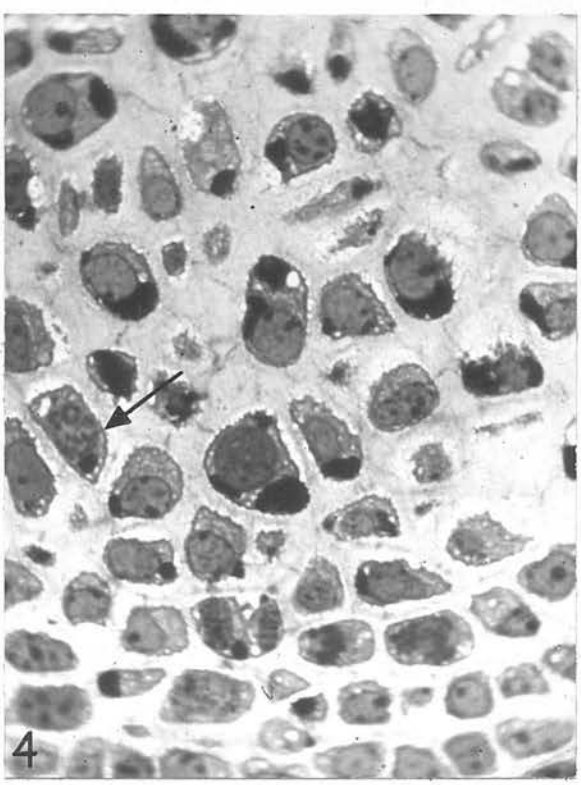
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(d) Late chondrogenic tissue (mouse, 18-day-old embryo). The chondroblasts are usually rounded or oval cells with an eccentrically placed nucleus. They differ considerably from the chondroblasts at a similar stage in the chick. The cytoplasm, after toluidine blue staining, shows one or two sharply defined areas which are less stainable than the rest of the cytoplasm; these areas appear bright red after PAS staining, indicating the presence of glycogen (plate 18, fig. 4). In addition very small hyaline vacuoles, representing the Golgi apparatus, can also be seen. The extracellular phase is extensive, fibrous and metachromatic when stained with toluidine blue. The chondroblasts, including those containing the glycogen deposits, are sometimes seen undergoing mitotic division. In paraffin sections of 16-day-old embryos, the mitotic figures may be observed also in the chondroblasts situated in the centre of various cartilage condensations of limbs (plate 19, fig. 2). The perichondrial cells are similar to the last stage.

(e) Epiphyseal cartilage (mouse, new-born). The chondrocytes from the epiphyseal cartilage of the third toe of the hind limb have a comparatively small, eccentrically placed nucleus (plate 19, fig. 1). The nucleocytoplasmic ratio, as in the case of chick, is approximately 0.5. The glycogen is extensive in amount and in addition to one or two large lumps, it is also present in a few smaller accumulations in the cytoplasm. The juxtanuclear Golgi apparatus area and at times large saccular dilations of the endoplasmic reticulum can also be seen in the light microscope. There is a very extensive fibrous extracellular phase, which, as in the chick, stains metachromatically with toluidine blue.

II Electron microscopic observations

(a) Mesenchyme (mouse, 12-day-old embryo). These cells are undifferentiated, as evidenced by the ultrastructure of various cell organelles.

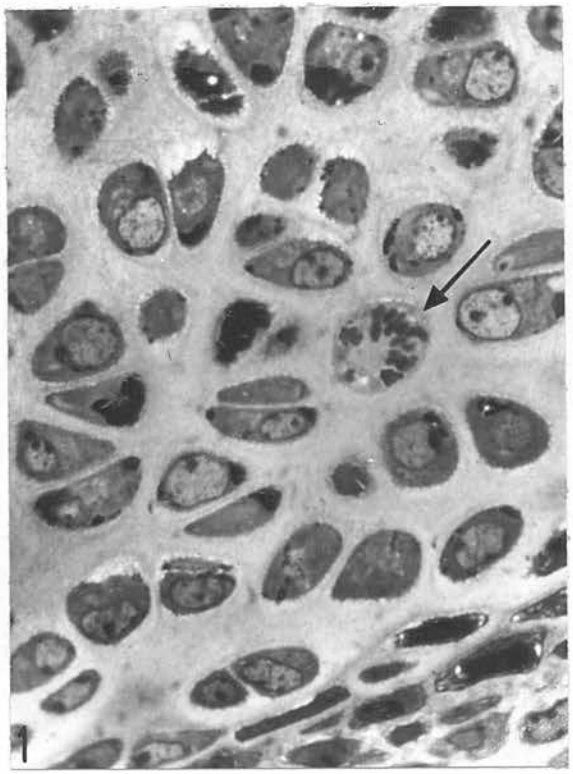
The nucleus is large, more or less rounded, centrally placed and with a slightly wavy outline (plate 19, fig. 3). The nucear envelope, around 37 mμ in thickness

Plate 19

Figure 1. Epiphyseal cartilage (mouse, new-born). This micrograph shows chondrocytes and a part of perichondrium. Notice the low nucleocytoplasmic ratio, eccentric location of the nuclei and a cell undergoing mitosis (arrow). M2, X 1,100.

Figure 2. Limb cartilage (mouse, 16-day-old embryo). This micrograph shows a mitotic figure (arrow) in the centre of cartilage, thus providing evidence to the view that even the fully formed cartilage grows by mitotic division of the chondrocytes. M7, x 400.

Figure 3. Mesenchyme (mouse, 12-day-old embryo). A low power electron micrograph showing a scarce extracellular phase (ep), large centrally placed nuclei (N) with wavy outline, and to some extent syncytial appearance of the tissue. M1, X 4,400.



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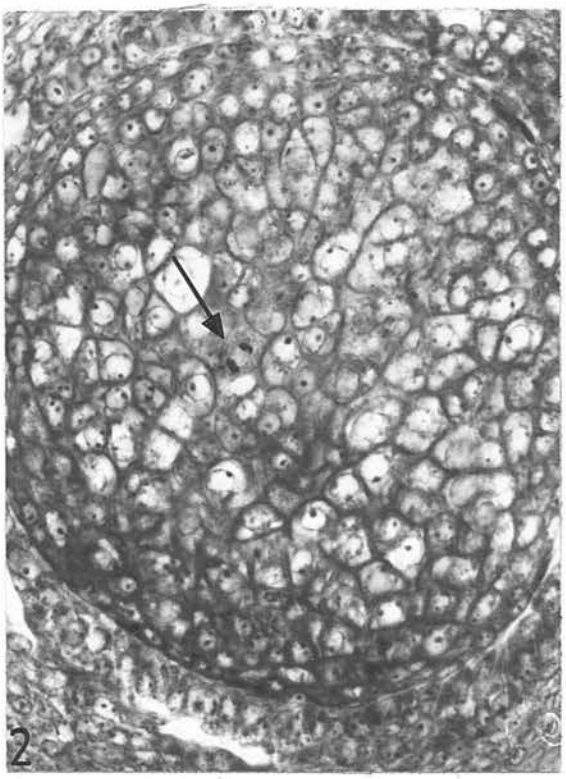

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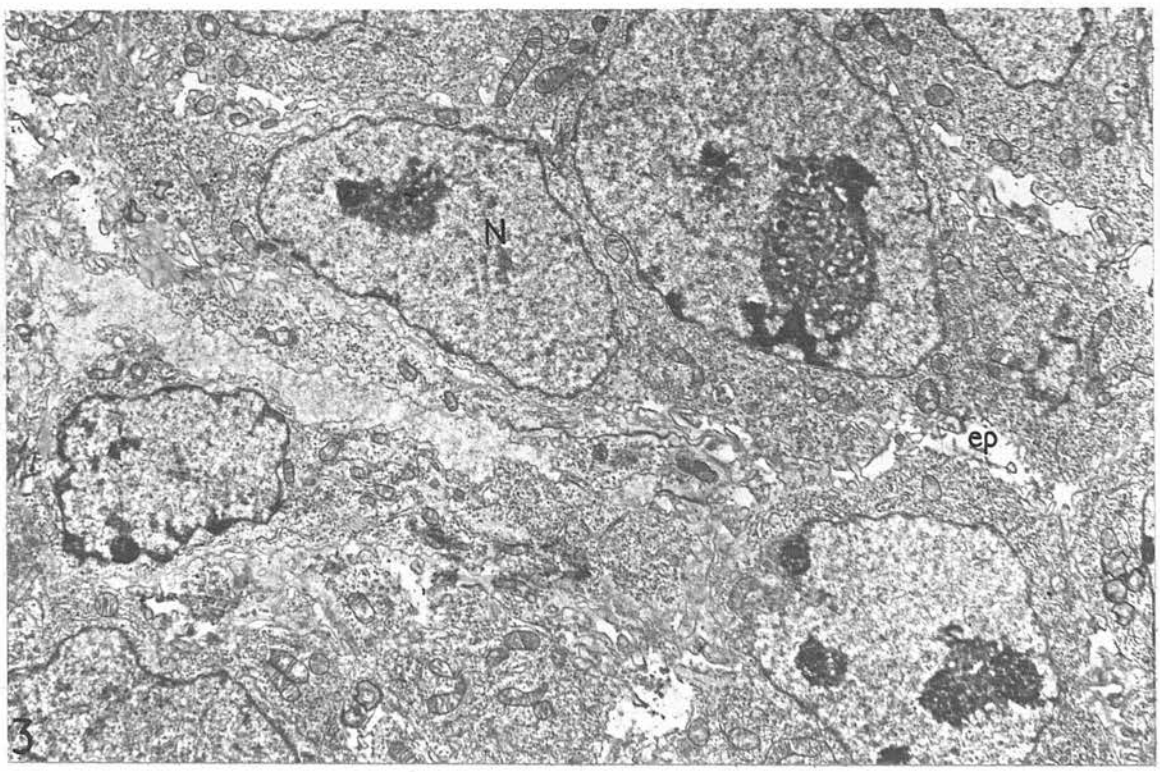

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(plate 20, figs. 1 - 3), consists of the usual outer and inner nuclear membranes with the perinuclear cisterna between them, and is frequently perforated with pores. The pores are filled with material whose electron density is either similar to or lower than that of the material on either side of the pore. The diaphragm, like that which bridges the pore in the nuclei of early chick mesenchyme cells, is either completely missing or indistinct. The outer nuclear membrane is not studded with ribosomes and only infrequently shows an incipient form of blebbing. The nuclear matrix consists of a homogeneous amorphous substance with electron dense granules of various sizes dispersed in it. In addition ribosome-like particles, around 20 - 28 mμ in diameter, are also often present. The appearance of the nucleolus is similar to that of the chick in corresponding stage.

The cytoplasmic ground substance is amorphous and slightly electron dense. It contains ribosomes, majority of which are present as polysomal groups of 4 to 10 ribosomes each.

The rough endoplasmic reticulum consists mainly of short cisternal profiles, around 70 mμ across (plate 20, fig. 1). Some smooth vesicular profiles, around 100 mμ in diameter, are seen very close to the cisternal profiles and are possibly part of the endoplasmic reticular system.

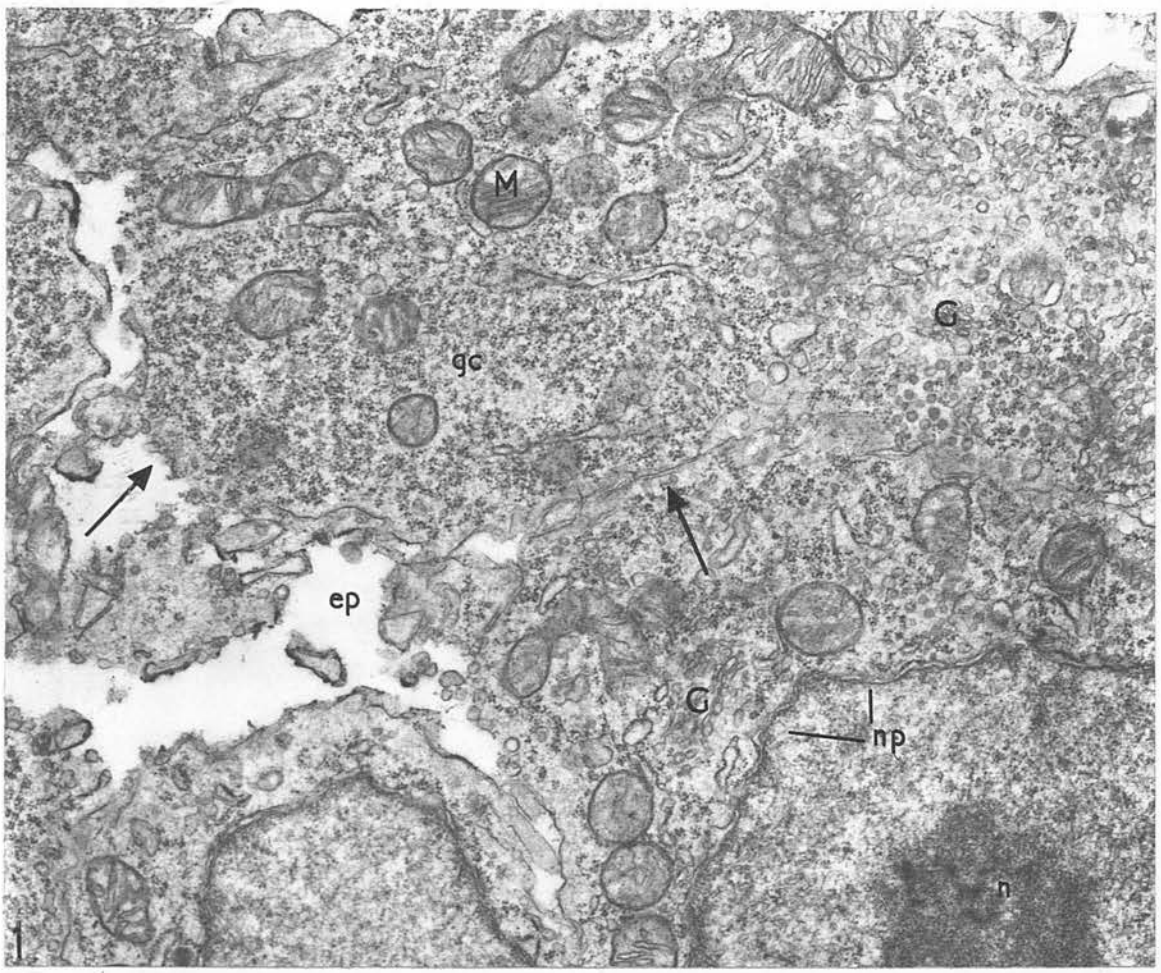
The Golgi apparatus, though inconspicuous, consists in sections of a very large number of rounded vesicles, around 85 mμ in diameter, and a large number of lamellar profiles, around 50 mμ wide and 170 - 500 mμ long; the latter are distributed in two to five groups (plate 20, figs. 1 - 3). The vacuoles are seldom seen and measure approximately 200 mμ. It appears as though the Golgi lamellae are in the process of formation by thinning, elongation and coalescence of the Golgi vesicles. The contents of both, the Golgi apparatus and the endoplasmic reticulum are amorphous, homogeneous and electron dense.

Plate 20

Figure 1. Mesenchyme (mouse, 12-day-old embryo). A general survey micrograph showing scarce and hyaline extracellular phase (ep), polysome-filled ground cytoplasm (gc), endoplasmic reticulum consisting of short cisternal and vesicular profiles, nuclei with a characteristic nucleolus (n) and nuclear pores (np), Golgi apparatus (G) in the process of formation and numerous mitochondria (M). The discontinuities of the plasmalemma are almost certainly artifacts, since while it is discontinuous at the sites of cell-to-extracellular phase junction, it is continuous at the sites of cell-to-cell attachment (arrows). M1, 20,700.

Figure 2. Mesenchyme (mouse, 12-day-old embryo). Part of a mesenchyme cell showing the lamellar (gl) and vesicular profiles of the Golgi apparatus, and also the cytosomes (cs, l and mv). M1, X 31,400.

Figure 3. Mesenchyme (mouse, 12-day-old embryo). A mesenchymal cell showing a typical Golgi apparatus (G), consisting of several groups of lamellae and vesicles, characteristic nucleolus (n) and an elongated structure (arrow) probably representing a cilium. M1, X 20,700.



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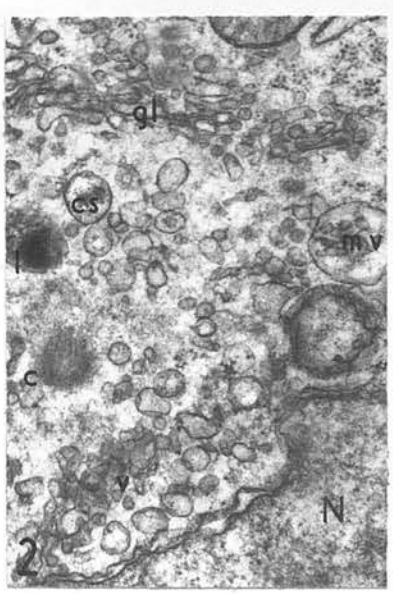

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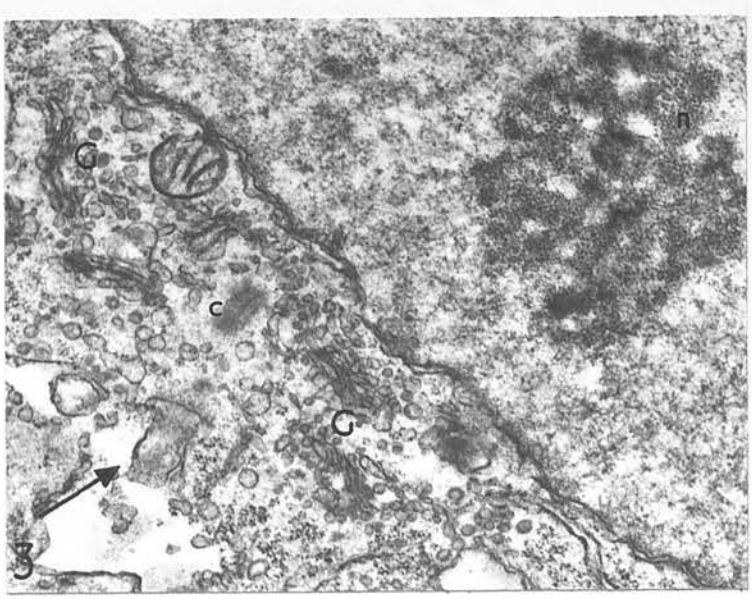

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The mitochondria are numerous and for that reason appear as the most prominent cell organelle (plate 20, fig. 1). They are usually circular in section, around 0.6 μ in diameter; some elongated mitochondria measuring up to 2.6 μ x 0.6 μ are also seen. The mitochondrial matrix is homogeneous and moderately electron dense. The mitochondrial granules, unlike those in the comparable chick tissue, are rarely present and are about 24 mμ in diameter. Cytosomes appear either as lipid droplets, about 290 mμ in diameter, or as multivesicular bodies, about 450 mμ in diameter (plate 20, fig. 2).

The plasmalemma, around 12 to 14 mμ thick in a cross section, is not seen as a continuous membrane all around the cell; however, it is probably a preservation artifact (plate 20, fig. 1).

The extracellular phase is scarce and electron translucent (plate 20, fig. 1).

(b) Prechondrogenic tissue (mouse, 13-day-old embryo). The cells show distinct signs of differentiation leading to cartilage. The relative development of its cellular organelles and the extracellular phase is remarkably different from that of the chick tissue.

The nucleus and the nucleolus of the prechondroblasts are very similar to those of the mesenchyme cells in 12-day-old embryos. The cytoplasmic ground substance, ribosomes, mitochondria and the Golgi apparatus also do not show any considerable changes (plate 21, figs. 1 and 3). The endoplasmic reticulum, however, is better developed than in the mesenchyme of 12-day-old mouse embryo. It is present as long convoluted granular cisternal profiles approximately 100 mμ across; these profiles sometimes dilate into saccular cisternae up to 320 mμ wide (plate 21, fig. 2). Their contents are homogeneous and moderately electron dense. The plasmalemma is a more or less continuous structure but, unlike that in the comparable chick tissue where it is continuous, it appears blurred in places (plate 21, fig. 3).

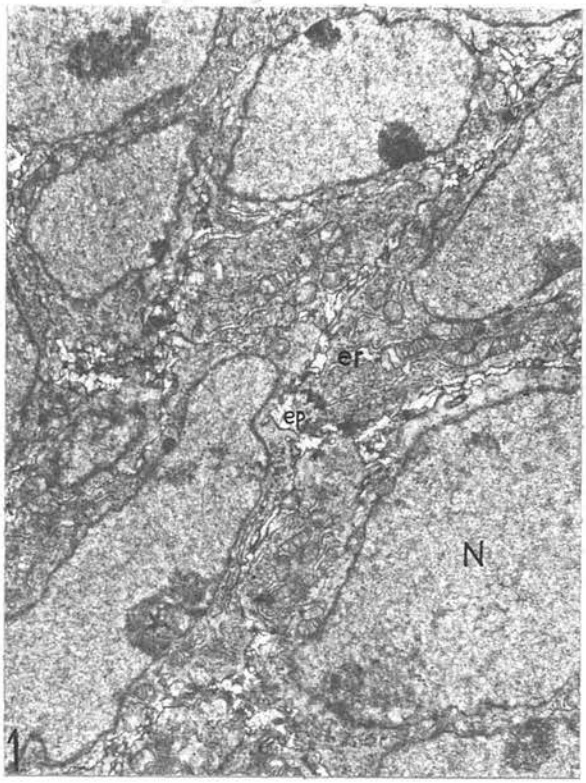
Plate 21

Figure 1. Prechondrogenic tissue (mouse, 13-day-old embryo). A low power electron micrograph showing that the amount of extracellular phase (ep) is scarce, and the general appearance of cells except the development of the endoplasmic reticulum (er) is similar to previous stage. M1, X 4,400.

Figure 2. Prechondrogenic tissue (mouse, 13-day-old embryo). The micrograph shows a typical cisternal profile of the endoplasmic reticulum (er), and also nuclear pores (np) and the Golgi apparatus (G). M1, X 20,700.

Figure 3. Prechondrogenic tissue (mouse, 13-day-old embryo). Compare the structure of these cells to those of plate 20, figures 2 and 3. There is a remarkable similarity. Also notice the plasmalemma (pl), which is blurred at places. M1, X 31,400.

Figure 4. Prechondrogenic tissue (mouse, 13-day-old embryo). Notice the fibres (f) and granules (g) of the extracellular phase. M1, X 72,400.



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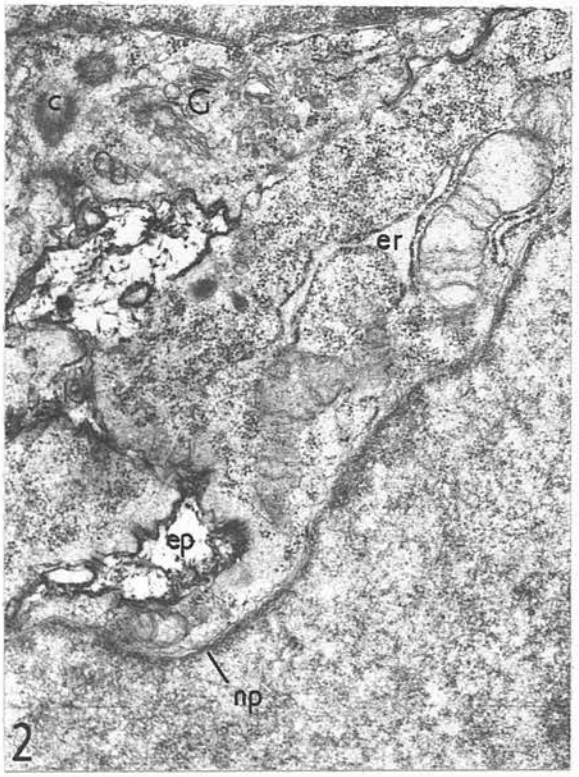

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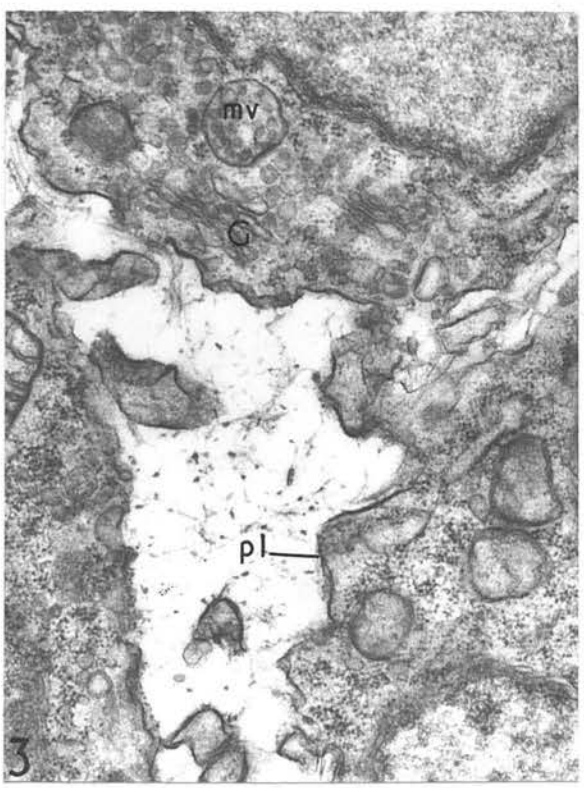

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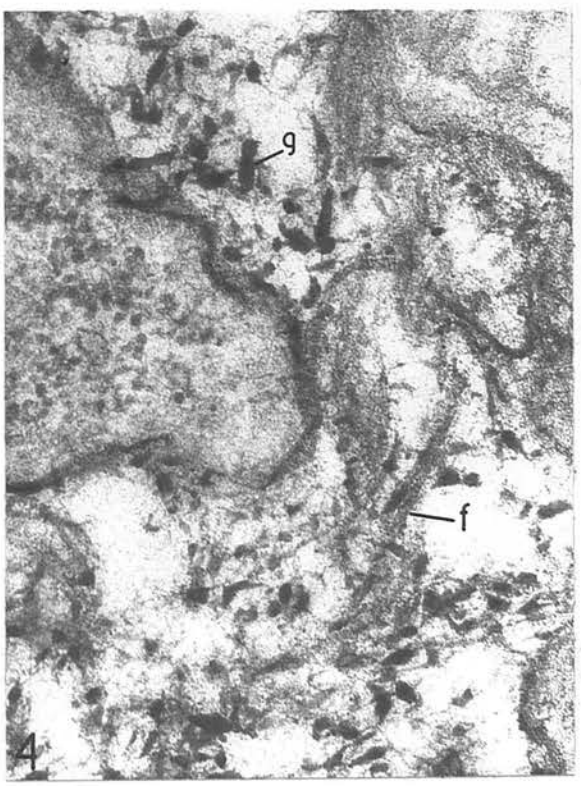


4

The extracellular phase, as compared with that of the previous stage studied, does not increase in amount, but contains the electron dense granules in addition to the fibres (plate 21, figs. 1 and 4). It is also in contrast to comparable stages during the differentiation of the chick cartilage where the granules are ~~found~~ missing. The fibres are around 16 mμ thick, unbanded, short and straight or slightly curved. They are sometimes very close to the plasmalemma and may even appear to merge with it. The electron dense granules are present either in association with the fibres or lie free between the fibres. They vary in diameter from 14 to 42 mμ but are usually around 31 mμ across.

(c) Chondrogenic tissue (mouse, 16-day-old embryo). The young chondroblasts differ from the prechondroblasts, both as regards the ultrastructure of the cells and the quantity of the extracellular phase.

The nucleus is irregular in shape (plate 22, figs. 1 and 5) and its matrix is moderately electron dense, homogeneous and contains ribosome-like particles, around 27 mμ in diameter. In addition there is considerable amount of chromatin which, in the interphase nuclei, consists of homogeneously dispersed particulate elements. The chromatin is usually distributed either in small lumps in the nuclear matrix or as approximately 100 mμ wide continuous layer which is situated just inside the nuclear envelope and is interrupted under the nuclear pores; some larger lumps which are either attached to the inner nuclear membrane or to the nucleolus can also be seen. The chromatin of mitotic nuclei, as compared to the interphase nuclei, is comparatively less electron dense (plate 22, fig. 1).

In the cytoplasm the endoplasmic reticulum is well developed and consists mainly of granular elongated cisternal profiles, around 100 mμ in diameter, and saccular cisternae, up to 0.48 μ wide. The Golgi apparatus remains virtually unchanged except that some vacuoles, up to 0.3 μ in diameter, are also present.

Plate 22

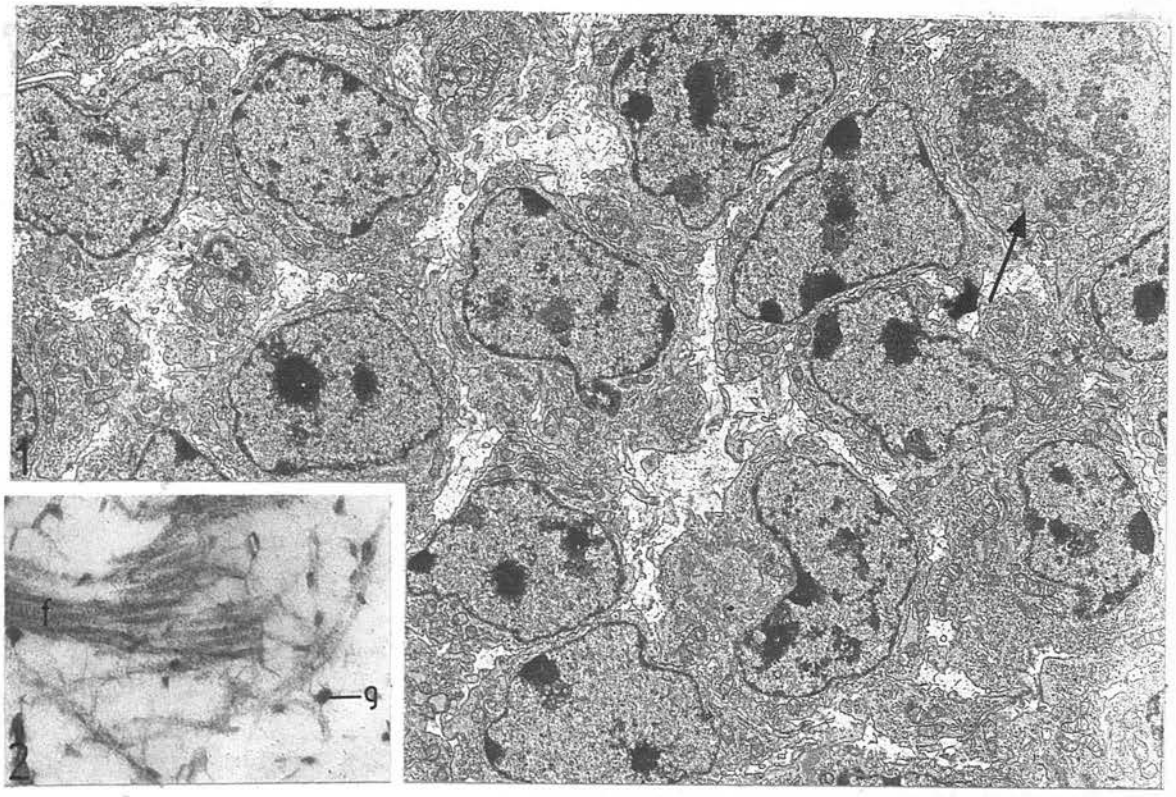
Figure 1. Chondrogenic tissue (mouse, 16-day-old embryo). A low power electron micrograph showing the general appearance of tissue, irregular shape of nucleus and the mitotic nucleus (arrow). Compare the electron density of mitotic chromosomes with the chromatin of interphase nuclei. M1, X 4,400.

Figure 2. Chondrogenic tissue (mouse, 16-day-old embryo). The extracellular phase showing the granules (g) and fibres (f). The fibres seem to have an incipient banding. M1, X 72,400.

Figure 3. Chondrogenic tissue (mouse, 16-day-old embryo). A cilium (arrow) is seen with its tip in the infolding of cellular membrane of another cell. This arrangement suggests that the cilia are not locomotory in function. M1, X 40,600.

Figure 4. Chondrogenic tissue (mouse, 16-day-old embryo). The micrograph shows a part of the plasmalemma. The 'infolding' (arrow) seen here may be either of pinocytotic nature or derived from endoplasmic reticulum. M1, X 31,000.

Figure 5. Chondrogenic tissue (mouse, 16-day-old embryo). A typical cellular structure of differentiating cells is apparent. Also compare the shape of nuclei to those of chick in plate 8 figure 1. M1, X 20,700.



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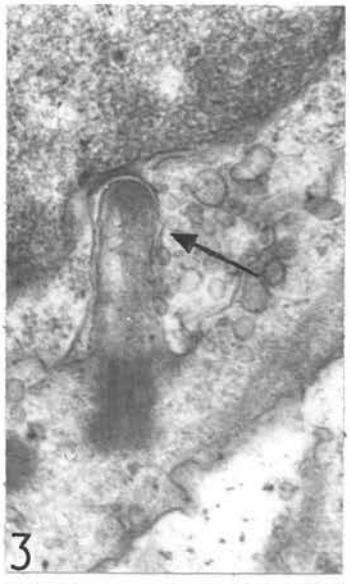

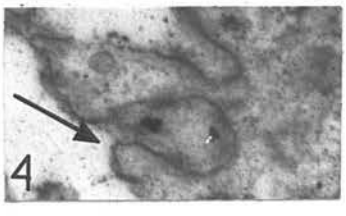

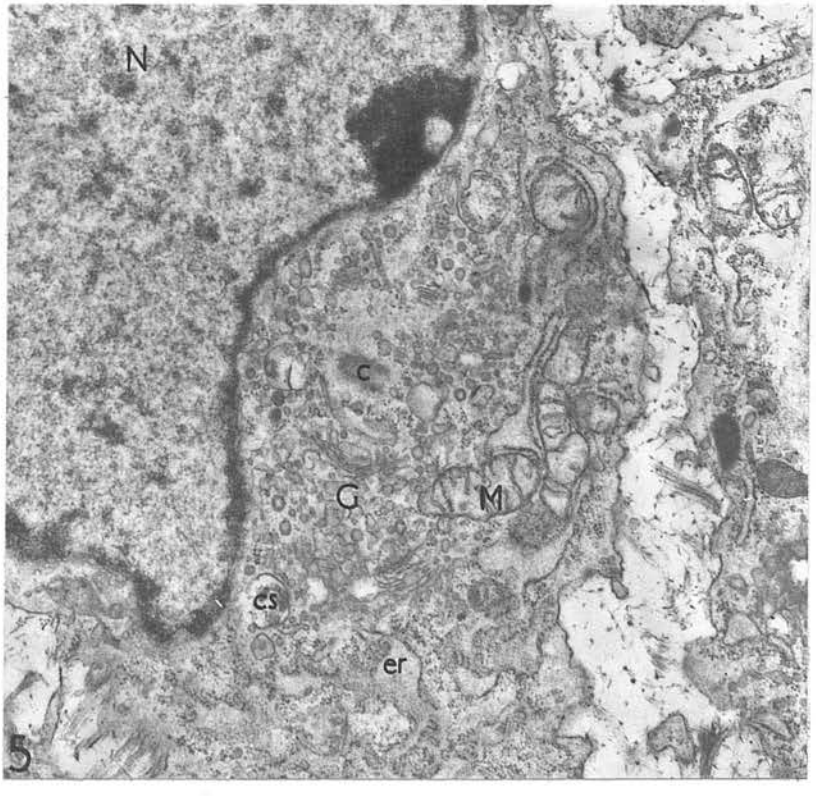
N
c
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The cytosomes, both the lipid droplets and the multivesicular bodies, are present in various sizes and sometimes are bounded by a double membrane. A cilium is sometimes noticed at this stage as well as in developing cartilage of 14-day-old embryos (plate 22, fig. 3). The cilium may be up to 1.2 μ long and 0.34 μ thick. Similar single cilia are reported by Scherft and Daems (1967) from chondrocytes of epiphyseal cartilage of embryonic mouse radii and also by Glauert et al. (1969) from articular chondrocytes of chick embryos. The plasmalemma, probably due to its very convoluted outline in vivo, does very often appear ill-defined when tangentially sectioned. Plate 22, figure 4 shows infoldings of the well defined plasmalemma which are filled with moderately electron dense material. They probably are either vesicles of pinocytotic origin or cytoplasmic vesicles opening out into the extracellular phase.

In the extracellular phase the individual fibres are short, more or less straight and around 14 mμ thick; there is a suggestion of an incipient banding pattern in the fibres (plate 22, fig. 2). The electron dense granules are common and usually measure about 17 mμ in diameter.

(d) Late chondrogenic tissue (mouse, 18-day-old embryo). The fully formed chondroblasts, as in the case of chick, have a scalloped appearance. Moreover, as compared with young chondroblasts, important changes can be noticed in the contents and the ultrastructure of these cells.

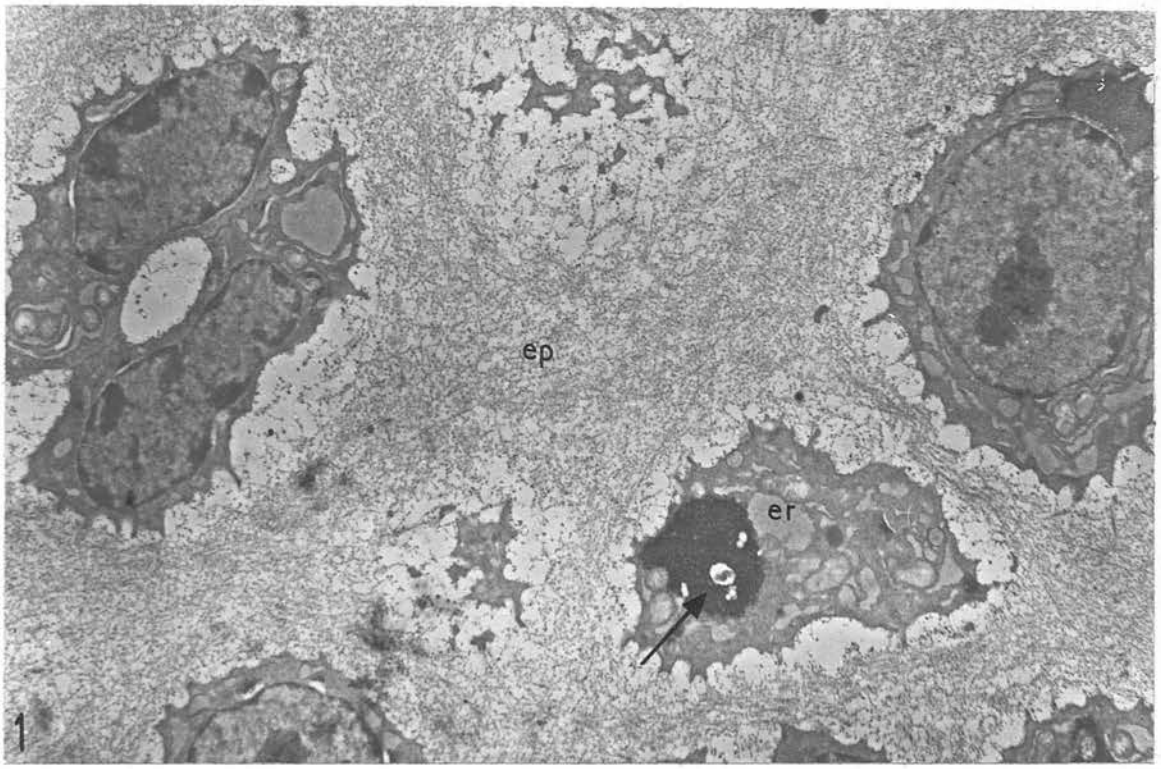
The nucleus is noticeably smaller and eccentrically placed. The chromatin is distributed as in the previous stage (plate 23, fig. 1). The nucleolus lies either free in the nuclear matrix or is attached to the nuclear envelope. It shows the usual structure: fibrillar regions enclosed in the particulate regions.

The cytoplasmic ground substance is moderately electron dense and is filled with polysomes. The mitochondria, though present as before, are no longer the

Plate 23

Figure 1. Late chondrogenic tissue (mouse, 18-day-old embryo). A low power electron micrograph showing an extensive extracellular phase (ep), with fibres and granules, and the cells, which in one instance have a cell-to-cell attachment. Endoplasmic reticulum (er) is the most prominent cell organelle, and the electron opaque glycogen may have some electron translucent spaces included in it (arrow). Distribution of chromatin is like one in the previous stage. M2, X 4,400.

Figure 2. Late chondrogenic tissue (mouse, 18-day-old embryo). Notice the Golgi apparatus (G) with its beaded and ordinary lamellae, vesicles and vacuoles; the granular structure of glycogen mass, and the mitochondria (M). M2, X 20,700.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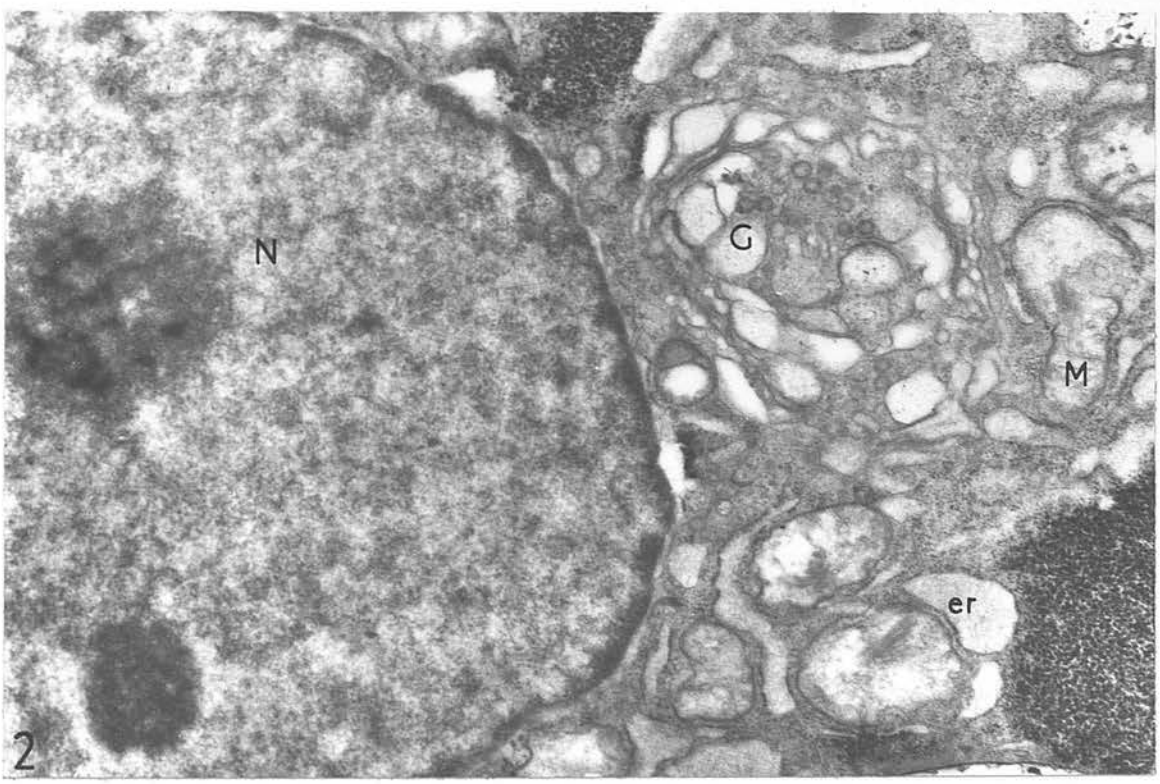
N
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prominent cellular organelle. Their matrix is moderately electron dense and only rarely contains any mitochondrial granules (plate 23, fig. 2).

The endoplasmic reticulum is the most prominent cell organelle (plate 23, fig. 1; plate 24, figs. 1 - 4). It consists mainly of an anastomosing network of elongated granular cisternal profiles around 150 mμ across, and saccular cisternae of differing dimensions, some of which are as large as 2.3 μ x1.8 μ. The saccular cisternae, though of various shapes, are seldom, if ever, regularly oval or rounded. The vesicular profiles of the endoplasmic reticulum are rare and most probably are cross-sections of the elongated cisternal profiles. The cisternae are frequently seen to approach the plasmalemma and fuse with it. Sometimes their contents are observed to be in direct continuity with the extracellular phase (plate 24, figs. 2 and 3).

The juxtanuclear Golgi apparatus consists of the lamellae, vesicles, and the vacuoles (plate 23, fig. 2). The lamellae, usually arranged in one or two groups, are of widely varying dimensions and shapes. They are to some extent beaded in appearance and are swollen towards their ends. The Golgi vesicles, 120 mμ in diameter, may be involved in the formation of the Golgi vacuoles, 450 mμ in diameter. Both the Golgi vesicles and the lamellae have amorphous contents and are bounded by smooth membranes. The Golgi vacuoles contain the fibrous and granular structures that are similar to the corresponding structures in the chondrogen granule of the Golgi vacuoles of the chick chondrocytes (plate 24, fig. 1). Sometimes they also contain, in addition, an amorphous moderately electron dense material. Quite a few of these vacuoles can be seen in the cortical area of the cytoplasm, suggesting their movement to the periphery and ultimate discharge of their contents into the extracellular phase. The distinction between the Golgi vacuoles and the saccular cisternae of the endoplasmic reticulum exists in the mouse in much the same way as in the chick.

Plate 24.

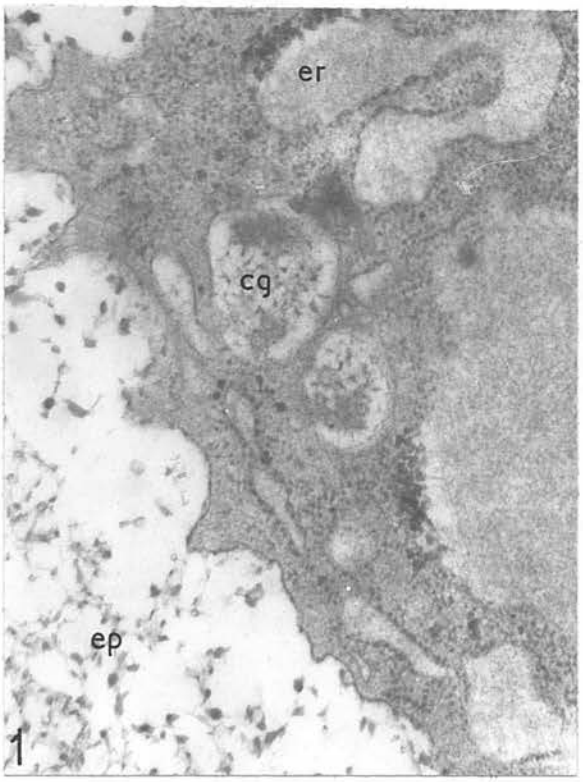
Figure 1. Late chondrogenic tissue (Mouse, 18-day-old embryo). Notice the substructure of chondrogen granule (cg), and also the well developed saccular cisternae of the endoplasmic reticulum (er). M2, X 40,600.

Figure 2. Late chondrogenic tissue (Mouse, 18-day-old embryo). Notice a direct connection between the cisternae of the endoplasmic reticulum (er) and the extracellular phase (arrow). M2, X 40,600.

Figure 3. Late chondrogenic tissue (Mouse, 18-day-old embryo). The saccular cisternae of the endoplasmic reticulum are well developed and one of them (er) appears to open into the extracellular phase (arrow). M2, X 20,700.

Figure 4. Late chondrogenic tissue (mouse, 18-day-old embryo). The substructure of glycogen granules, after periodic acid-lead citrate treatment, is visible. One of the granules is in the extracellular phase (arrow). Also note the electron translucent spaces inside the glycogen and the well developed endoplasmic reticulum (er). M4, X 40,600.

Figure 5. Late chondrogenic tissue (Mouse, 18-day-old embryo). The extracellular phase showing fibres (f) and granules. (g). M2, X 108,000.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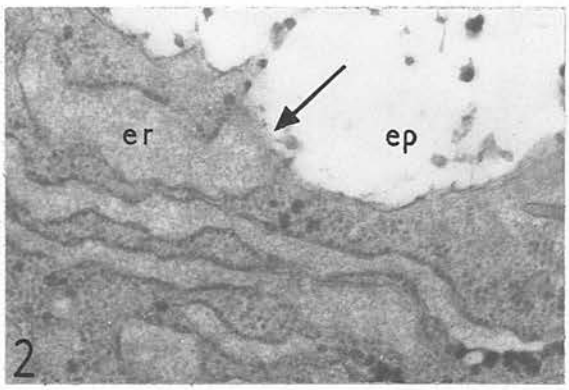
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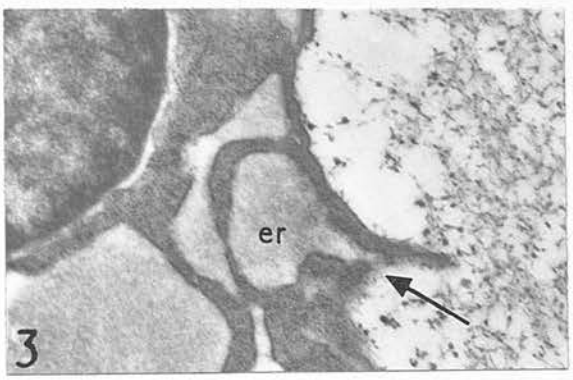
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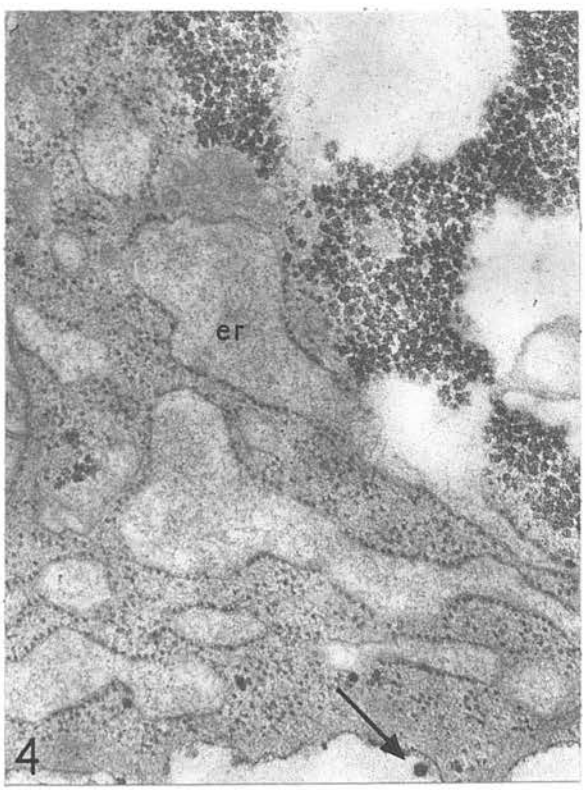
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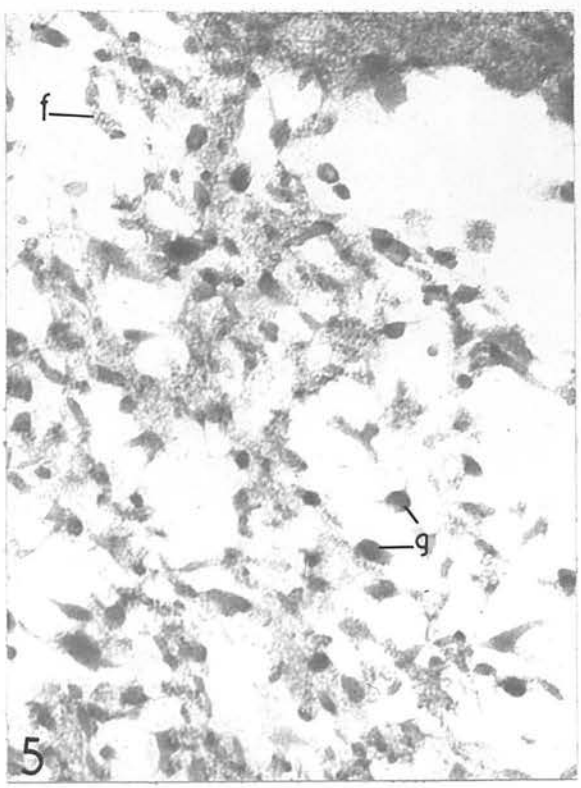
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The presence of glycogen in the late chondroblasts of the mouse epiphysis most clearly distinguishes them from those of the chick. The main mass of glycogen, seen also in the light microscope after PAS staining, is present in the cortical cytoplasm as one or two large accumulations unbounded by any membrane. It is also present in smaller amounts, either as single granules or as granule-groups scattered in the cytoplasm. Sometimes the glycogen granules, like the ribosomes, appear to be arranged on the endoplasmic reticulum or outer nuclear membranes; the arrangement is probably a mere spatial phenomenon and is not a reflection of functional association as suggested by Manasek (1968). The glycogen granules are never seen in the Golgi vacuoles or cisternae and very rarely a granule is seen in the extracellular phase (plate 24, fig. 4).

The extracellular phase is extensive (plate 23, fig. 1; plate 24, fig. 5). The fibres are short, straight and unbanded, around 18 mμ thick. The electron dense granules, around 23-37 mμ in diameter, are polygonal in shape and are usually associated with the fibres. It appears that there are relatively more granules in the extracellular phase of the mouse cartilage as compared to that of the chick.

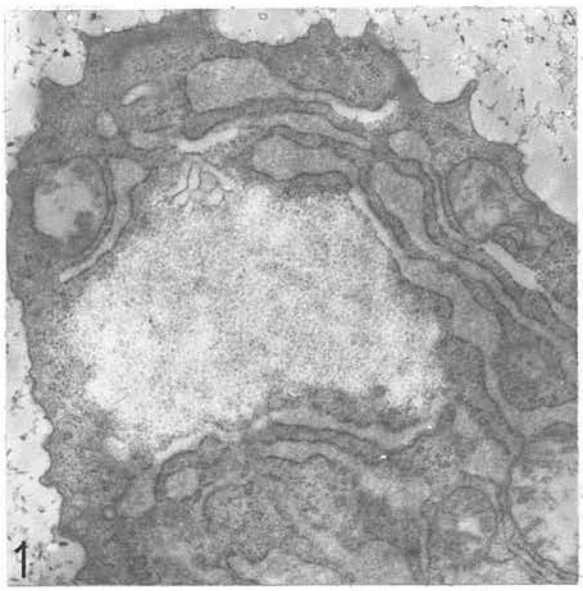
(e) Epiphyseal cartilage (mouse, new-born). The normal chondrocytes are usually separated from each other, but on rare occasions even a cell-to-cell attachment can be seen. These cells, like those in the chick, are regular in shape and lack large indentations (plate 25, fig. 3).

The nucleus endoplasmic reticulum, Golgi apparatus and the mitochondria of the chondrocytes are usually similar to those of the chondroblast (plate 26, figs. 1 - 4). A cilium has also been seen occasionally (plate 26, fig. 4). The cytosomes are fairly frequent. The structure of most of them suggests that they originate from the Golgi apparatus since their ultrastructural appearance

Plate 25

Figures 1 and 2. Late chondrogenic tissue (Mouse, 18-day-old embryo). These two adjacent sections of the same cell mounted on the same grid, show the difference in the electron density of the glycogen-containing area after exactly the same treatment. M2, X 20,700.

Figure 3. Epiphyseal cartilage (Mouse, new-born). Note the small eccentric nuclei, shallow indentations on cell surface, extensive extracellular phase, distinction between the area of Golgi apparatus (G) and endoplasmic reticulum (er), and widespread distribution of glycogen. M2, X 4,400.



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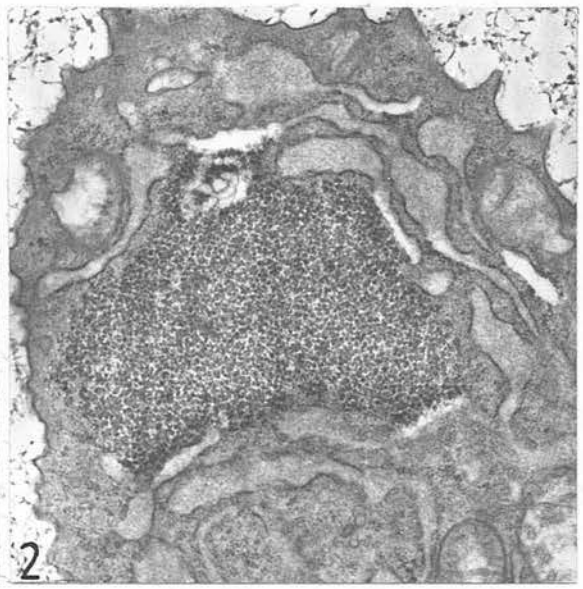

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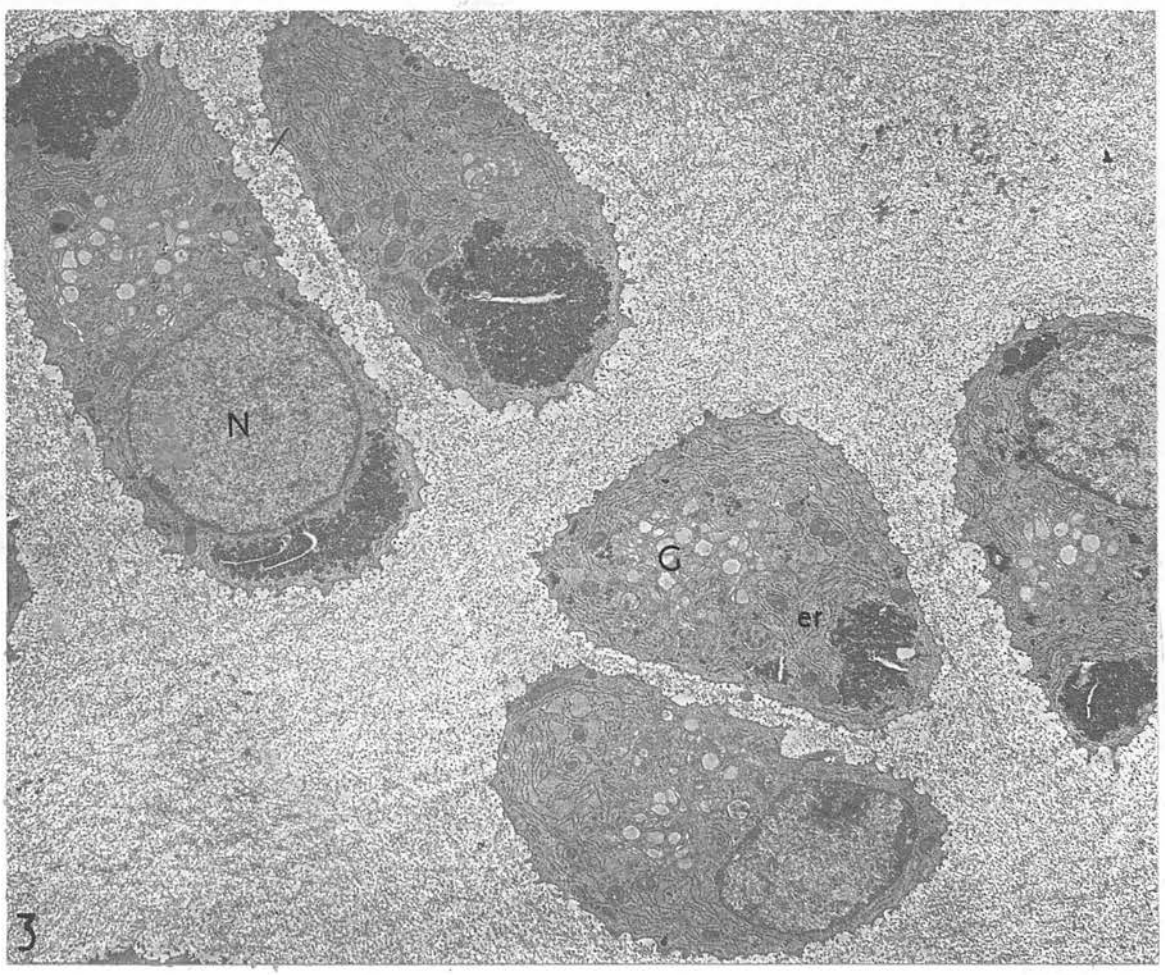

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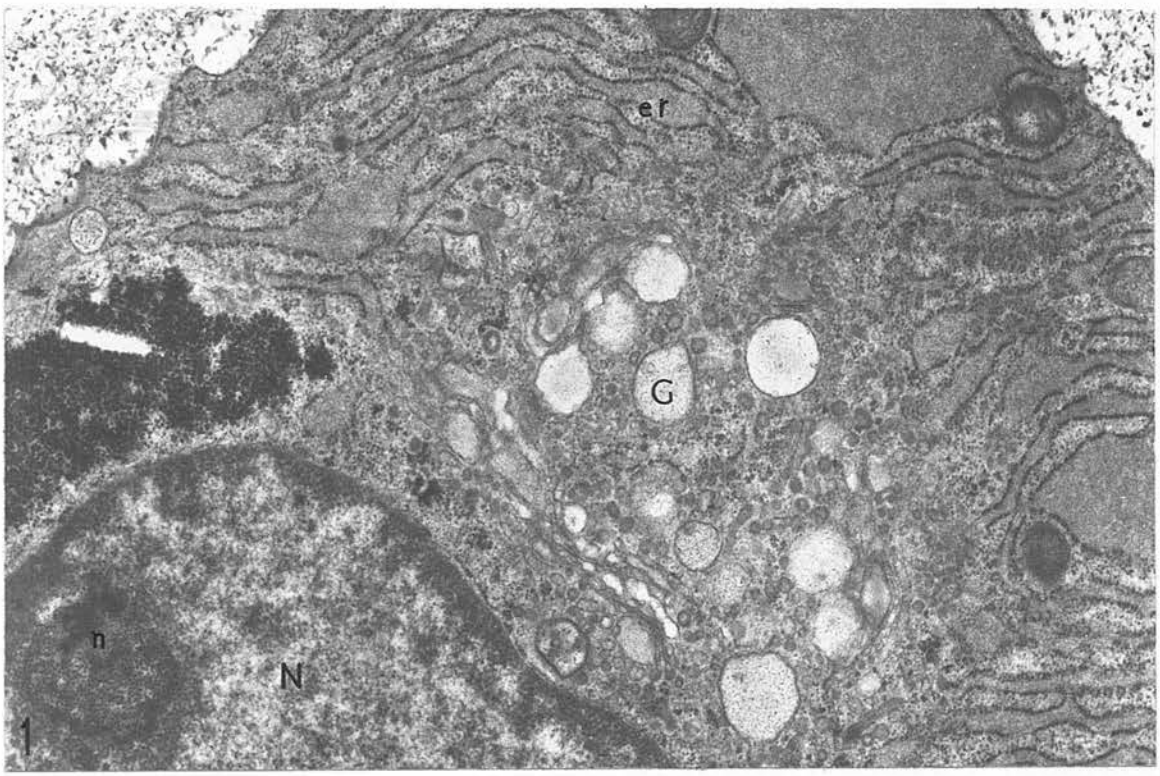
Plate 26

Figure 1. Epiphyseal cartilage (Mouse, new-born). The endoplasmic reticulum (er) is well developed. The Golgi apparatus (G), nucleus (N) and nucleolus (n), also show typical structure. M2, X 20,700.

Figure 2. Epiphyseal cartilage (Mouse, new-born). Notice a direct connection between perinuclear and endoplasmic reticular cisternae (arrow). A typical nucleolus, attached to the inner side of nuclear envelope, is also seen. M2, X 20,700.

Figure 3. Epiphyseal cartilage (Mouse, new-born). The plasmalemma and the membrane of endoplasmic reticulum cisternae are fused to each other (arrow). M2, X 56,000.

Figure 4. Epiphyseal cartilage (Mouse, new-born). The micrograph shows a cilium (arrow), Golgi apparatus (G) and a cytosome (cs). M2, X 20,700.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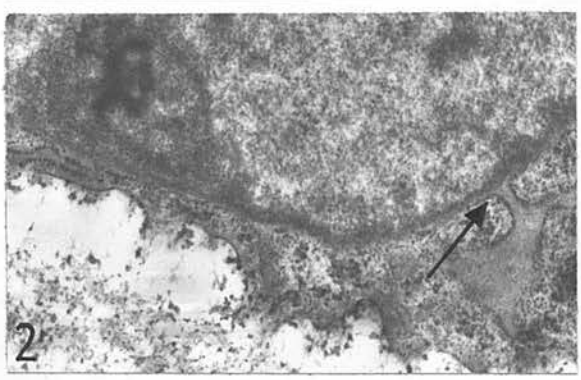
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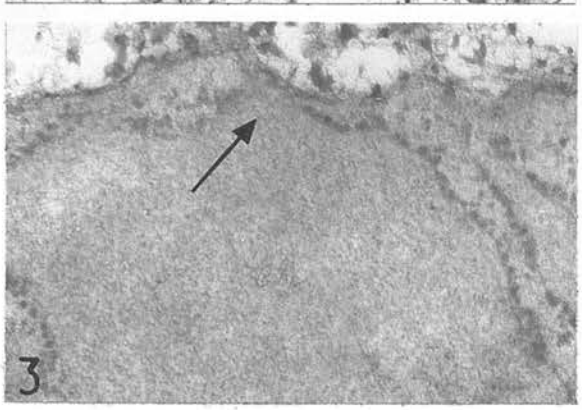
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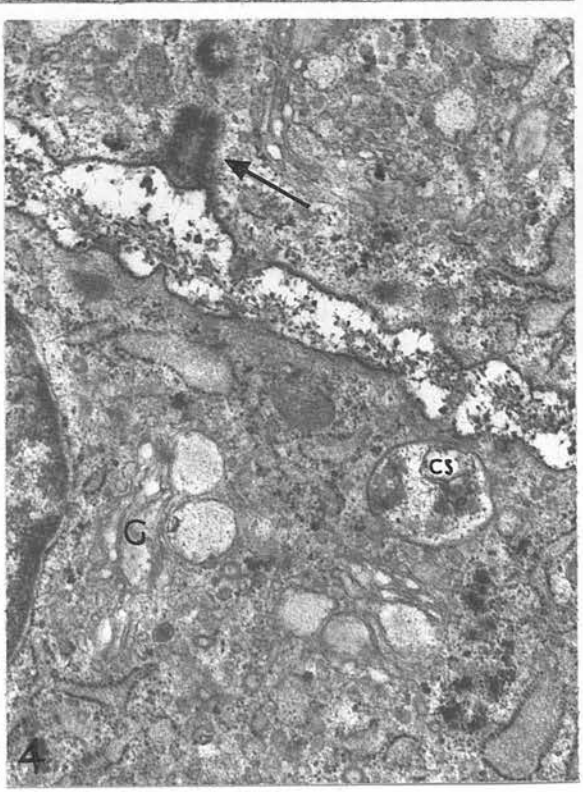
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4

is very similar to the Golgi vacuoles except that they contain a few round vesicles as well as electron dense materials and membraneous structures. Some cytosomes appear in the form of lipid droplets and measure about 330 mμ in diameter.

The amount of glycogen is increased as compared with that in the cartilage of 18-day-old mouse embryo. Its distribution, however, is similar to that in the 18-day-old embryo since the main mass of the glycogen is present in the cortical cytoplasm and smaller accumulations, consisting of up to a few hundred granules, are interspersed between, though never within, the various cell organelles.

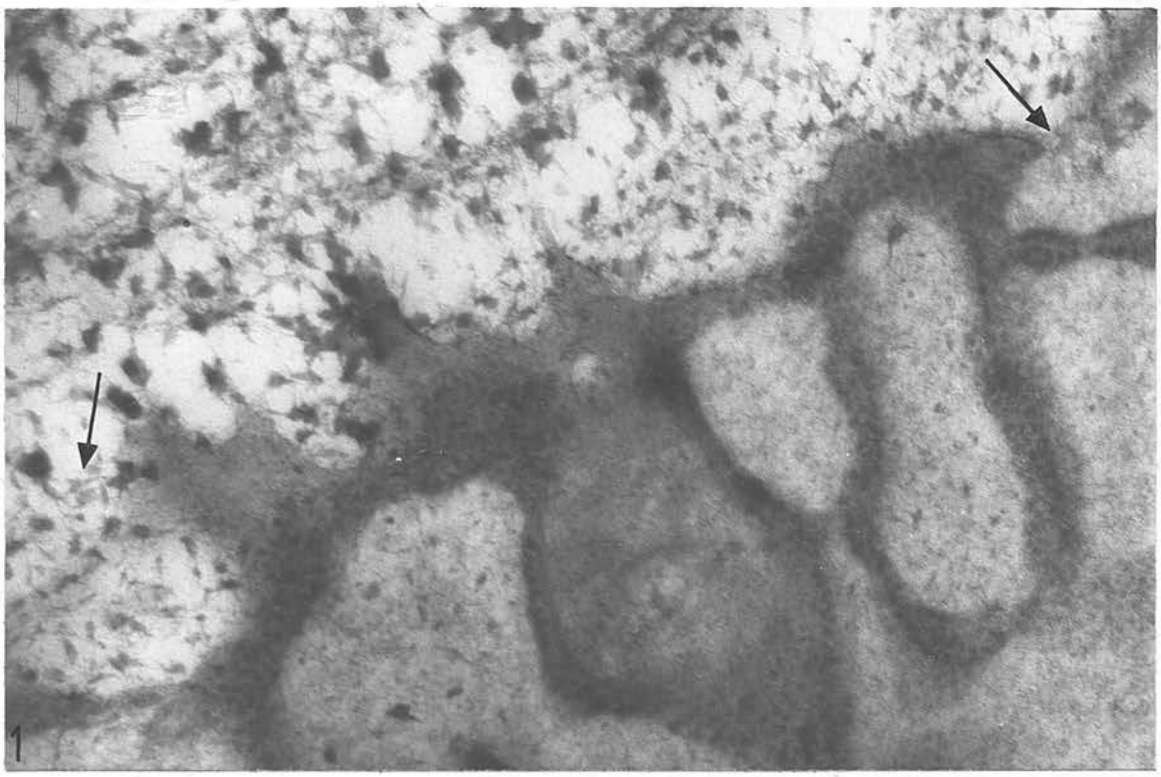
The extracellular phase consists of a ground substance with very many fibres and polygonal granules embedded in it (plate 28, fig. 1). The majority of the fibres are short and straight measuring about 18 mμ in thickness. Some, however, extend for a considerable distance. Each fibre consists of two, or sometimes three, parallel fibrils whose ultrastructure suggests an incipient periodicity of 5.5 mμ, due to the presence of small electron dense particles, approximately 2 mμ across, alternating with electron translucent spaces, 2.5 mμ wide. Associated with the fibres or lying interspersed between them are large numbers of polygonal granules which measure around 20 x 35 mμ.

The peripheral chondrocytes, that is those lying just under the perichondrium, are considerably different from the centrally placed chondrocytes (plate 27, figs. 1 and 2). The overall ultrastructural appearance of these cells is for some unknown reasons much inferior than that of the centrally located chondrocytes; however, it is most likely that these are the hypertrophied cells. The nucleus has a convoluted outline and appears very compact due to the relatively electron dense nuclear matrix. The chromatin is almost electron opaque and is distributed in clumps, and as a thick layer just inside the inner nuclear membrane, except

Plate 27

Figure 1. Epiphyseal cartilage (Mouse, new-born). A cell undergoing hypertrophy. Notice the saccular cisternae of the endoplasmic reticulum, sometimes connected with the extracellular phase (arrows), and the increased electron density of ground cytoplasm. M2, X 56,000.

Figure 2. Epiphyseal cartilage (Mouse, new-born). A hypertrophying chondrocyte showing an increase in the electron density of ground cytoplasm and nucleus, prominently saccular nature of the endoplasmic reticulum (er). A well developed Golgi apparatus (G), absence of chromatin below the nuclear pores (np), and presence of electron-opaque glycogen (arrow). M2, X 20,700.



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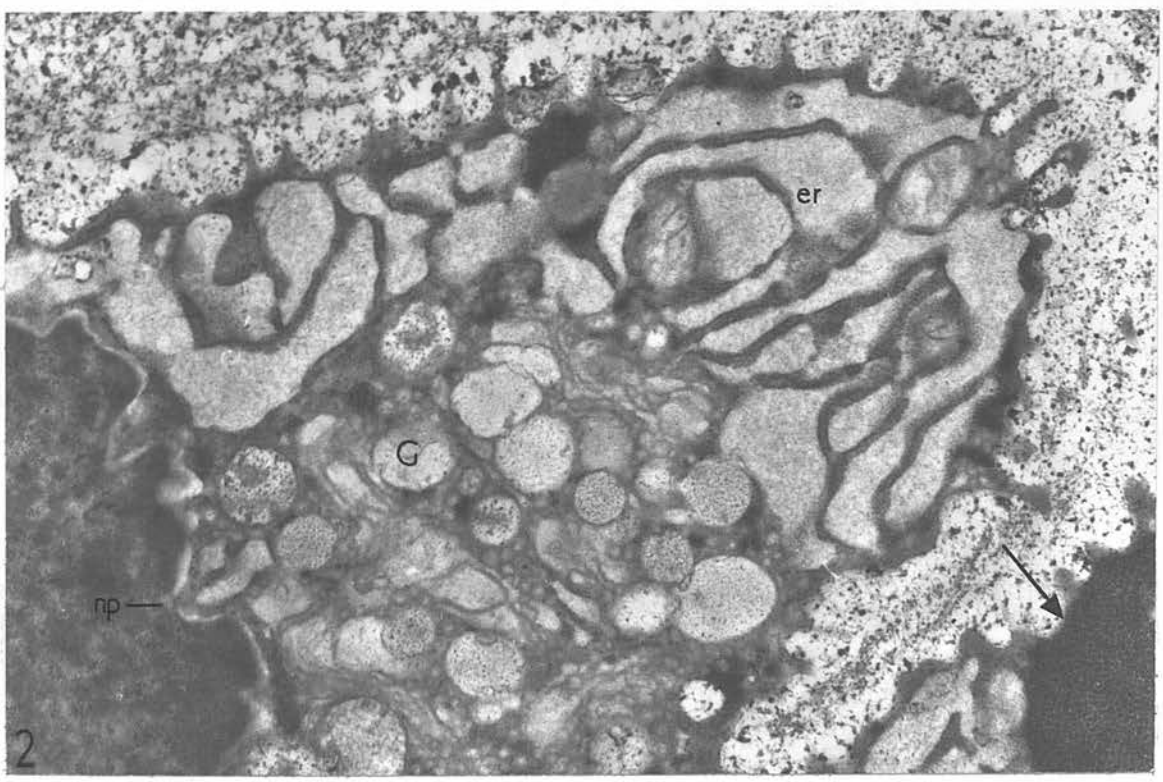

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below the nuclear pores (plate 27, fig. 2). The nuclear membrane cannot be properly made out but an electron lucent perinuclear cisterna of varying width is present. The ground cytoplasm is scarce and very electron dense. The well developed endoplasmic reticulum seems to occupy most of the cellular space and is largely similar to that of the centrally placed chondrocytes; but, it consists mainly of saccular cisternae and only a few elongated cisternae are present, (plate 27, fig. 1). The juxtanuclear Golgi apparatus is compactly packed but has all of its three usual components, though the vesicles are comparatively few in number. The mitochondria are usually enclosed within the endoplasmic reticulum network. The increasing amount of glycogen also occupies considerable space in the ground cytoplasm; it appears electron opaque in the low power electron micrographs.

4. CYTOCHEMICAL INVESTIGATIONS OF GLYCOGEN IN CARTILAGE

I. Demonstration of glycogen

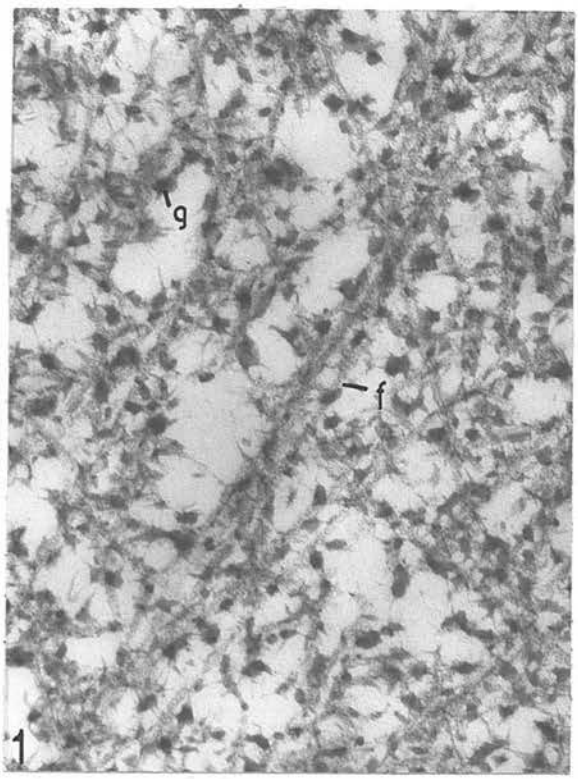
For the demonstration of glycogen Araldite embedded material was used for both light and electron microscopy. In the light microscope glycogen appears bright red after PAS-reagent staining and turns to magenta after counterstaining with toluidine blue. With toluidine blue alone it stains light blue in contrast to the deep blue of the ground cytoplasm. In low power electron micrographs the glycogen is seen in the cytoplasm as lumps of electron opaque material which at higher power can be resolved into granular units. These units are characteristic of glycogen and are distinguishable from the ribosomes not only because of their larger size, 25-50 mμ as compared to 20 mμ, but also due to their substructure after oxidation with periodic acid and staining with lead citrate, as also shown by Perry (1967). The substructure, visible only after this treatment, shows each granule to consist of 10 to 25 smaller subunits of approximately 5 mμ in diameter (plate 24, fig. 4).

Plate 28

Figure 1. Epiphyseal cartilage (Mouse, new-born). The extracellular phase contains fibres (f) and granules (g); some fibres seem to have a faint periodicity of 5.5 mμ. M2, X 72,400.

Figure 2. Late chondrogenic tissue (Mouse, 18-day-old embryo). The glycogen from these cells has been removed by digestion with amylase (arrows). M9, X 1,600.

Figures 3 and 4, Late chondrogenic tissue (mouse, 18-day-old embryo). Adjacent sections from the same block, showing the similarity in the intracellular distribution of PAS-positive material in light micrograph and periodic acid-lead citrate reactive material in the electron micrograph. M2, X 2,500 and X 9,000, respectively.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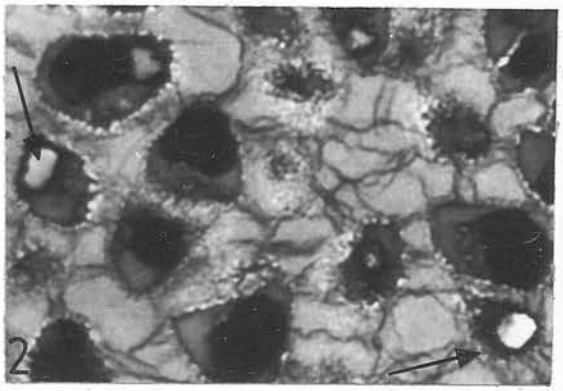
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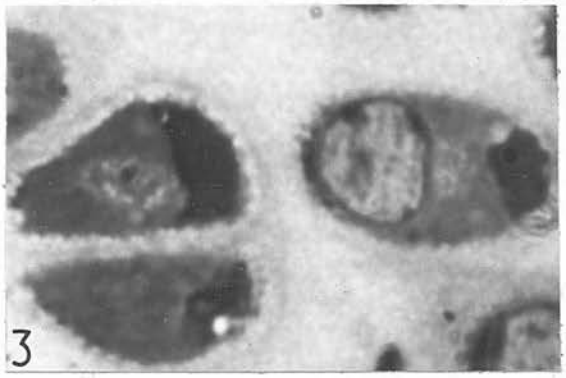
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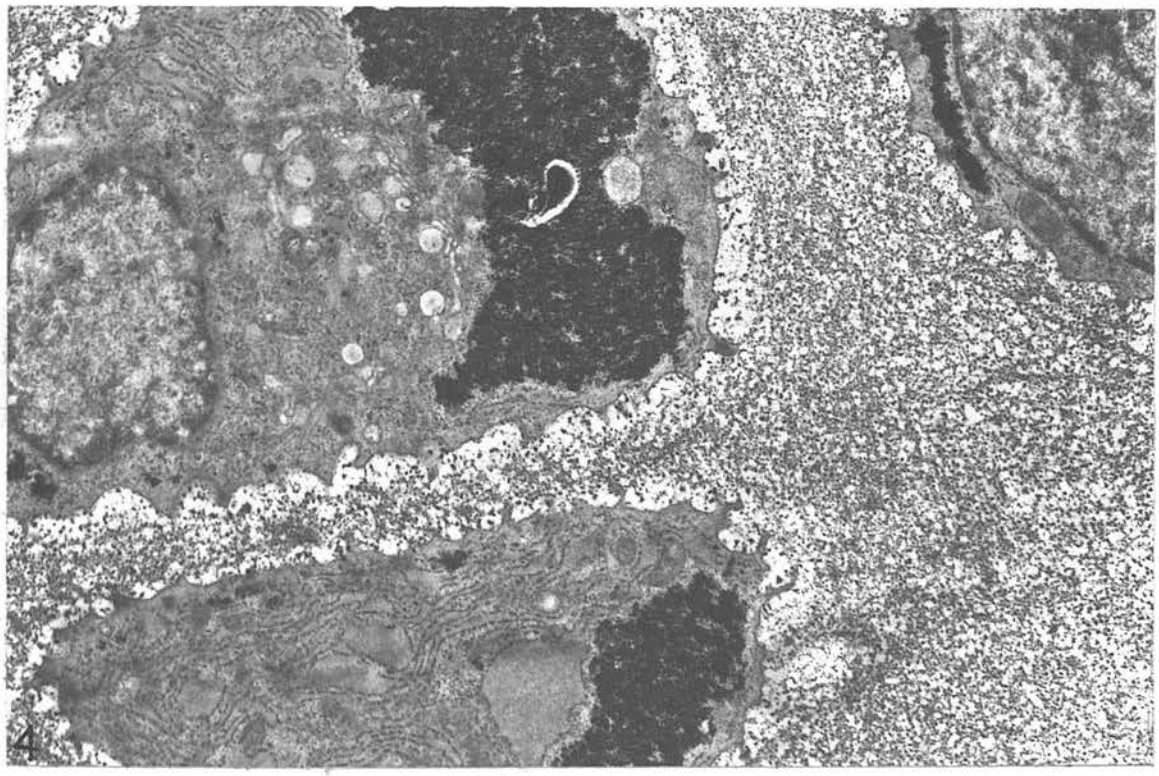
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The following procedures were adopted to confirm that the PAS-positive material seen in the light microscope is identical with the material identified in electron microscopy after periodic acid-lead citrate treatment, and is glycogen. Thin sectioning for electron microscopy was followed by cutting approximately 1μ thick sections for light microscopy. In this way adjacent sections of the same cells could be examined by electron and light microscopy. The comparative study of these thick and thin sections, after appropriate treatments and staining, showed that the PAS-positive regions corresponded precisely with regions containing material reactive to periodic acid-lead citrate treatment (plate 28, figs. 3 and 4). For biochemical identification of glycogen (see Pearse, 1960) enzymatic digestion was carried out with α-amylase to show that the PAS-positive material in thick sections, and the electron opaque material in the thin sections is glycogen. In the glutaraldehyde-osmium tetroxide fixed material, the sections digested with the enzyme showed a considerably weaker PAS-positive reaction as compared with those without enzyme digestion; while the sections of the material fixed only with osmium tetroxide, become PAS-negative after the enzyme digestion (plate 28, fig. 2). This indicates that the PAS-positive material is glycogen.

The method of fixation in case of glycogen is considered to be very important since unsuitable fixation leads to polarization and clumping of glycogen (Mancini, 1948; Pearse, 1960). The freeze-drying technique is considered to preserve the glycogen in its <u>in vivo</u> state and results in the even distribution of glycogen throughout the cell (Mancini, 1948). It may be pointed out here that with the present method of fixation (with glutaraldehyde and osmium tetroxide) no polarization and artificial clumping seems to have occurred. In none of the electron micrographs does the glycogen appear to have displaced any of the other cytoplasmic organelles and it is distributed in unpolarized masses of various sizes as well as individual granules around 25-50 mμ in diameter. In a number of cases, however, the

areas that contained glycogen, as judged by the PAS-positive reaction of the adjacent 1μ thick light microscopic section, are seen not to be opaque but rather translucent. Similar electron microscopic appearance of glycogen is described by other workers (Godman and Porter, 1960; Silberberg, 1968, among others). The cause of this is uncertain but a fortunate observation on two adjacent thin sections of the same cell on the same grid showed that the electron opaque material is present in one section while it appears extracted in the adjacent section (plate 25, figs. 1 and 2). Another feature of glycogen accumulations is the frequent presence of electron transparent spaces inside the accumulations (plate 23, fig. 1; plate 24, fig. 4; see also Anderson, 1964).

II. Distribution of glycogen

(a) Chick The presence of glycogen in epiphyseal cartilage from the toes of the chick hind limbs was not detected in the present study at any time during chondrogenesis up to stage 39, regardless of the fixative used. However, some chondrocytes, from or near that part of the diaphysis where periosteal ossification has started, show slight amounts of glycogen distributed more or less evenly in the cytoplasm.

(b) Mouse The glycogen can be detected in the developing epiphyseal cartilage from the hind limb toes, as early as the chondroblast stage. The amount of glycogen increases as the chondrogenesis proceeds and is extensive in the hypertrophic chondrocytes. The precise intracellular distribution of the glycogen is described, both at the light and electron microscopic level, in the appropriate sections (see pages 64 and 76).

5. AUTORADIOGRAPHIC STUDY OF CARTILAGE USING PROLINE-H^3 IN TISSUE CULTURE

For autoradiographic examination at the light and electron microscope level the cartilage was incubated in the presence of proline-H^3 for various periods from 15 minutes to 24 hours. From these experiments, the cartilage incubated with tritiated proline for 1 hour gives the first positive light microscope autoradiograph after an exposure time of 40 days. In these light autoradiographs a few silver grains are scattered over the cell, while no grains are present over the extracellular phase: but no clear distinction could be made between the distribution of grains over nuclei and cytoplasm. In the corresponding electron autoradiographs, however, it is clear that the grains are present both over the cytoplasm and the nucleus (plate 30, fig. 1; see also Revel and Hay, 1963, and Israel, Salpeter and Steward, 1968 for distribution of grains over nucleus). After an incubation of 2 hours the cytoplasm and nucleus become well labelled, but the extracellular phase still remains essentially unlabelled (plate 29, figs. 1 and 2). The extracellular phase shows presence of the label after the cartilage has been incubated with proline-H^3 for 4 hours and is extensively labelled after an incubation time of 24 hours (plate 29, figs. 3 and 4). The density of the grains is always higher over the cells as compared with the extracellular phase.

These observations show that the proline is first incorporated by various components within the cells and then released into the extracellular phase, almost certainly being bound in the collagen protein. The passage of macromolecules containing proline-H^3 through the various cell organelles could not be demonstrated in the present study. For example, the electron autoradiographs of cartilage incubated with tritiated proline for 4 hours show grains scattered over the cytoplasm without any specific localization being apparent (plate 30, fig. 2). It is also worth noticing that the concentration of the grains over the peripheral cells of the cartilage nodule, as compared to the chondroblasts, is relatively

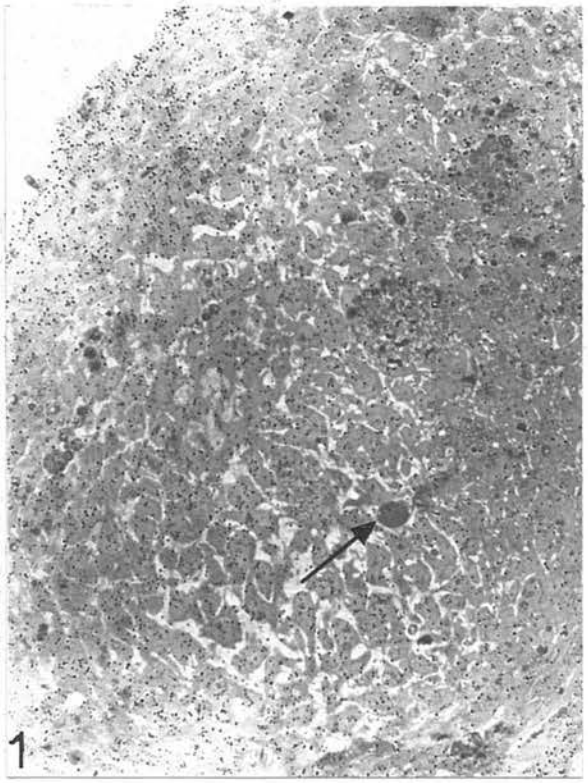
Plate 29

Figure 1. Chick cartilage differentiated in tissue culture, and incubated with proline-H^3 for 2 hours. The micrograph shows the distribution of grains almost completely on chondrocytes, and also a higher density of grains towards the periphery of nodule than towards the centre. The exposure time was 15 days. M1, X 400.

Figure 2. A high power light micrograph of some area shown in figure 1. Notice the virtual absence of grains from extracellular phase, and also from over a non-cartilage cell which is also indicated in figure 1 (arrow). M1, X 1,600.

Figure 3. Chick cartilage differentiated in tissue culture, and incubated with proline-H^3 for 24 hours. The micrograph shows a heavy labelling of the extracellular phase, and also the greater density of grains towards the periphery rather than the centre of condensation. Exposure time 3 days. M1, X 3,80.

Figure 4. A high power electron micrograph of the same tissue as shown in figure 3. It shows a very clear distribution of grains over the cellular as well as extracellular phase. Compare it to figure 2. M1, X 2,500.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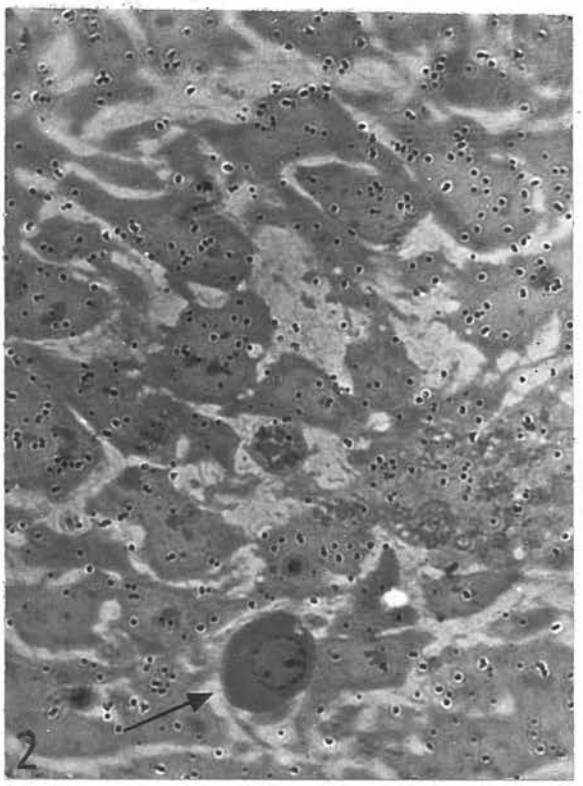
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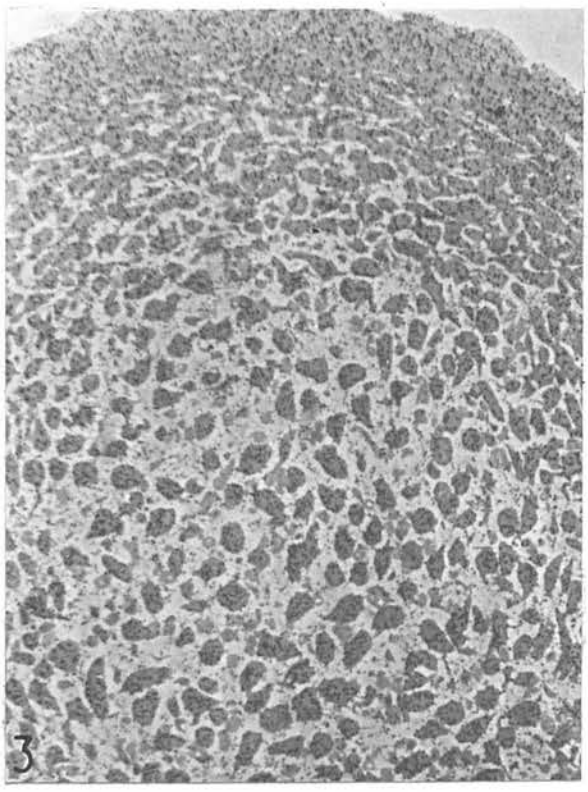
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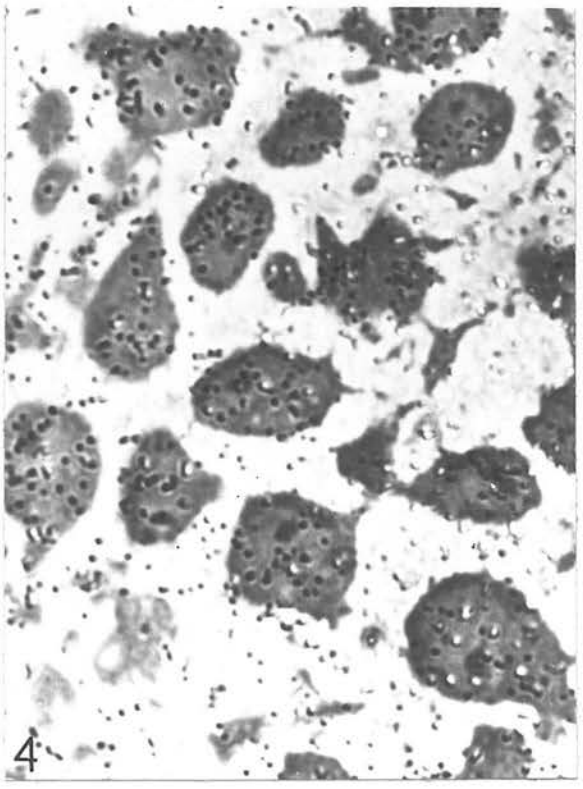
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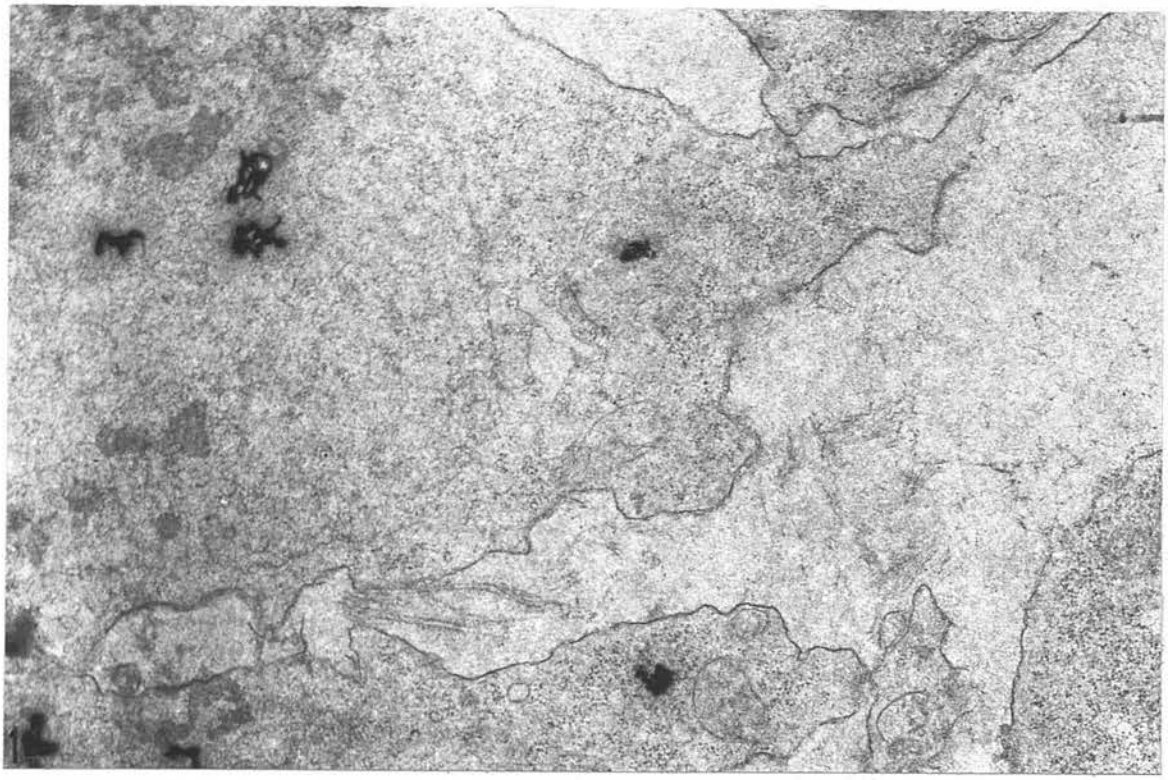
Plate 30

Figure 1. Chick cartilage differentiated in tissue culture and incubated with proline-H^3 for 1 hour. The micrograph shows the incorporation of label in the cytoplasm of the cells but not in the extracellular phase. Exposure time 8 months. M1, X 20,700.

Figure 2. Chick cartilage differentiated in tissue culture and incubated with proline-H^3 for 4 hours. The micrograph shows a very heavy but unspecific labelling of the cell by proline-H^3. Exposure time 2 months. M1, X 20,700.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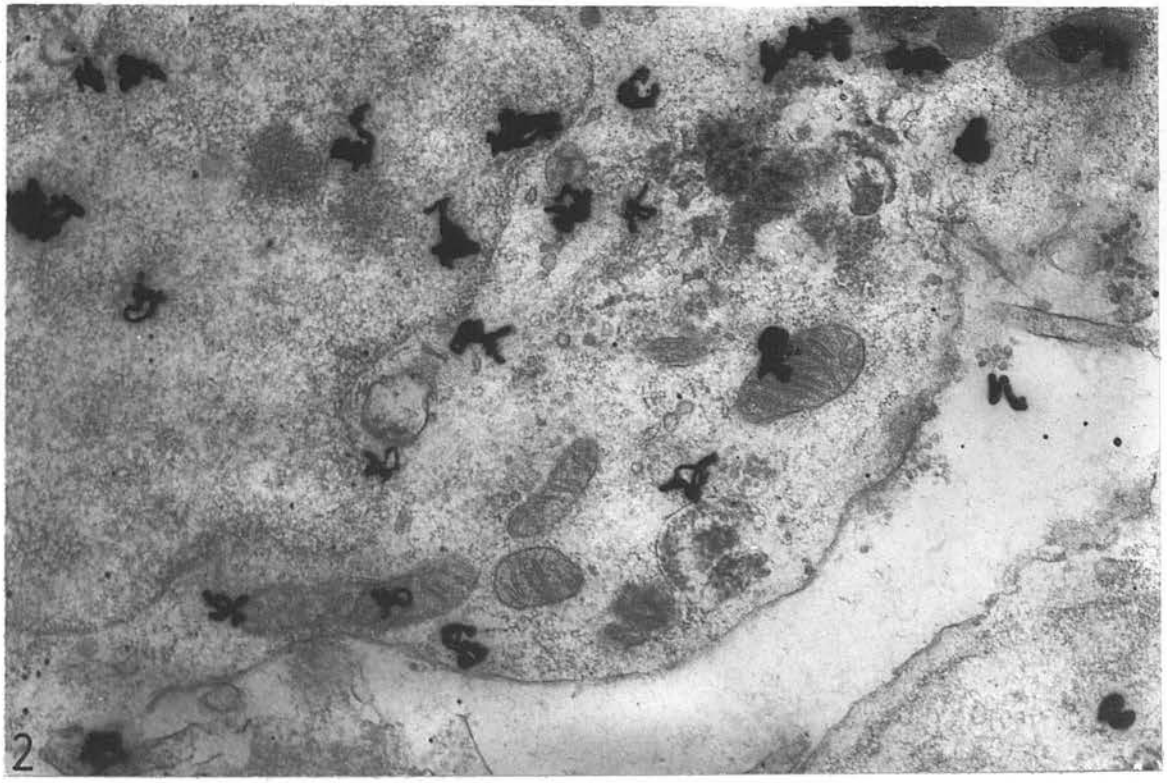
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higher (plate 29, fig. 1). Similar results, on chick limb cartilage grown in vivo, were reported by Deuchar (1963).

6. EFFECTS OF HYDROCORTISONE ON CHICK EMBRYOS

I. Macroscopical observations

Chick embryos incubated for 4 days were injected in ovo with 7.5 mg of hydrocortisone acetate per egg and examined after a further incubation of 24 hours. Control embryos were injected with 0.9% saline solution. The survival rate of the experimental and control embryos was as follows.

	Number of injected embryos	Number of surviving embryos	% of survivors
Experimental embryos	144	67	47
Control embryos	43	42	98

Some of the surviving embryos showed a slight retardation in development and limb defects in the form of haemorrhage and micromelia (plate 31, figs. 1-4; plate 33, figs. 7 and 8). The developmental retardation was noticeable in two ways; first, the experimental embryos were usually of stage 25 to 25+, while controls were of stage 25 to 26; and second, the experimental embryos weighed less than the controls.

	Number of embryos	Average weight per embryo
Experimental embryos	49	0.117 gm
Control embryos	24	0.146 gm

14 embryos (21% of the survivors) showed one or more of the limb defects listed below:

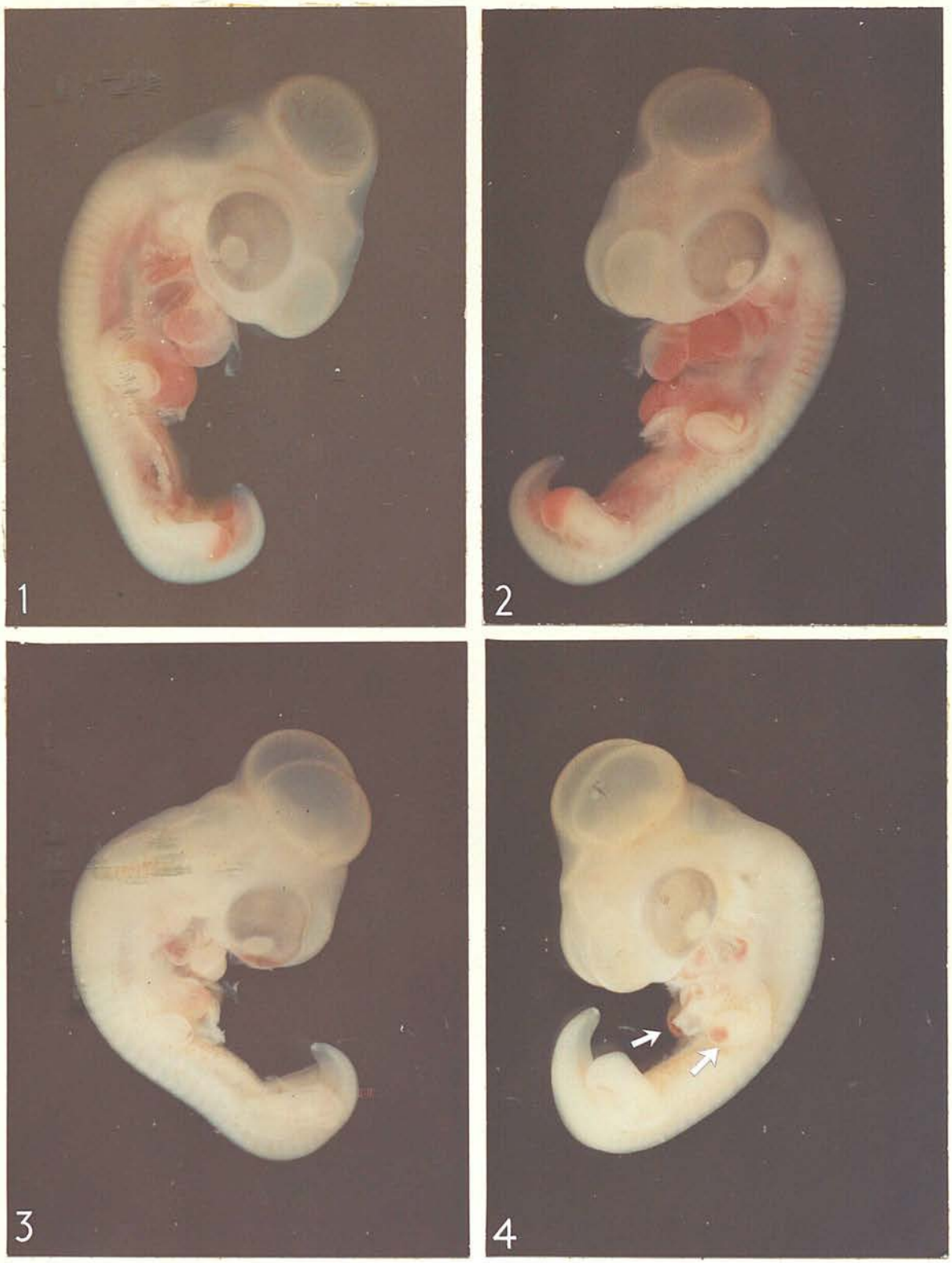
Limb defect	Number of embryos
I. Haemorrhage	
(a) Fore limb	8
(b) Hind limb	9
II. Micromelia	4

Plate 31

Figures 1 and 2. Right and left side views of a 4-day-old chick embryo injected with 7.5 mg of hydrocortisone and incubated for further 24 hours. The embryo had haemorrhage in three of the four limb-buds. Compare the extent of haemorrhage in fore and hind limb-buds in figure 1. Embryos photographed while in glutaraldehyde fixative. X 5.8.

Figure 3. This chick embryo, treated in the same way as that in figures 1 and 2, shows haemorrhage of only right fore limb-bud, and also of the optic area. X 5.8.

Figure 4. This chick embryo was also treated in the same way as those in figures 1 and 2. It showed haemorrhage in both fore limb-buds (arrows) but not in hind limb-buds. X 5.8.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The haemorrhage may be present in one or more limb-buds, without any apparent preference for fore or hind limb-buds or left or right side (plate 31, figs. 1-4). It varies widely in extent, as shown by comparison of fore and hind limb-buds in plate 31, figure 1. The haemorrhage appears in the apical area of the limb-bud; but a thin layer of healthy tissue was always present on the outer margin of the haemorrhage. This layer of tissue has been identified as ectoderm by histological examination. In addition the haemorrhage in tail (4 embryos) and head region (2 embryos) was also noted in some of the embryos that had a limb haemorrhage as well as in some others (3 embryos) that did not show any limb haemorrhage. Moreover, sometimes a general haemorrhage affecting the whole of one side of the embryo was seen. However, haemorrhages do not seem to be the only cause of death of the embryos, because some of the dead embryos did not show any sign of haemorrhage.

II. Light microscopic observations

A histological examination of limb-buds, from embryos injected with hydrocortisone acetate, usually reveals necrotic changes of mesoderm regardless of the macroscopically visible damages. It is a characteristic feature that the ectodermal lining along with the basement membrane remains unaffected regardless of the extent of necrosis or haemorrhage subjacent to it (plate 33, fig. 3).

The 3-day-old embryos, after an injection of 2 mg or more of hydrocortisone per egg and a further incubation of 24 hours, show necrosis, particularly in the limb mesenchyme. The necrotic areas are usually located in the central and apical regions of the mesenchyme in either one or both pairs of limbs. Sometimes necrosis can be seen also in the middle of dorsal or ventral side of the limb mesenchyme. The necrotic areas may or may not be in the close vicinity of the blood vessels.

Plate 32

Figure 1. A cross section of a 4-day-old chick embryo injected with 7.5 mg hydrocortisone and incubated for further 24 hours. Both the hind limb-buds show haemorrhage in the apical area. M6, X 32.5.

Figure 2. Limb-bud of an embryo treated similar to the embryo in figure 1. The haemorrhage in the apical mesoderm is accompanied by a very extensive necrosis in the central mesoderm. M7, X 110.

Figure 3. Similar to figure 2, but note the compact packing of blood cells in the haemorrhage. M7, X 160.

Figure 4. Limb-bud of an embryo treated similar to the embryo in figure 1. There is no haemorrhage or dilation of blood vessels, but necrosis is present in apical and central mesoderm. M7, X 130.

Figure 5. Similar to figure 4, but from an embryo showing extensive necrosis of apical mesoderm. The cell and nuclear debris are also seen in the intercellular space. Note the healthy state of overlying ectoderm. M7, X 250.

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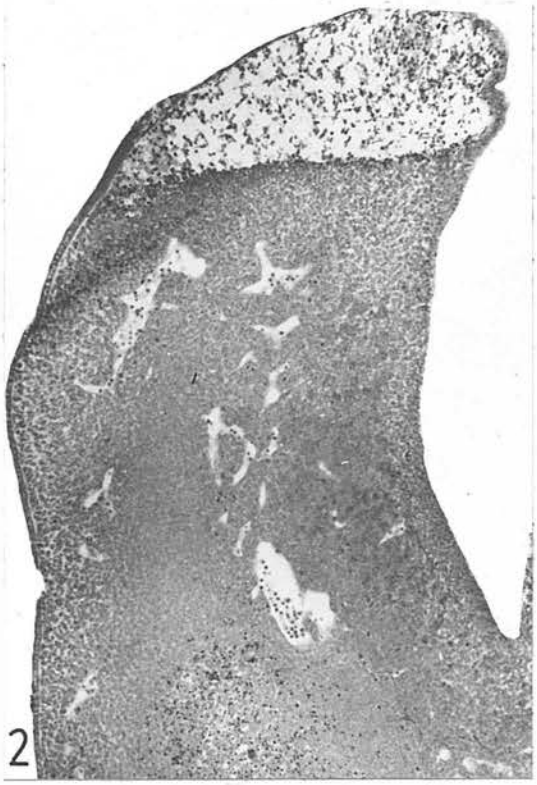

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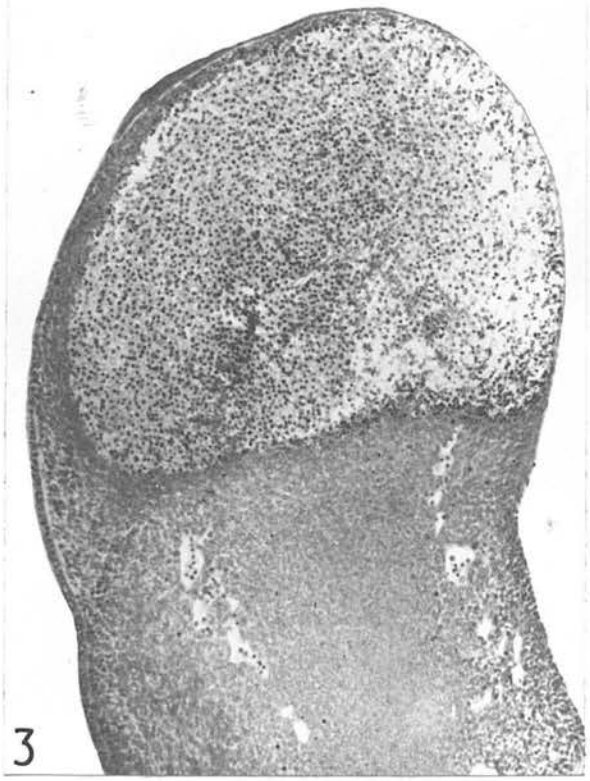

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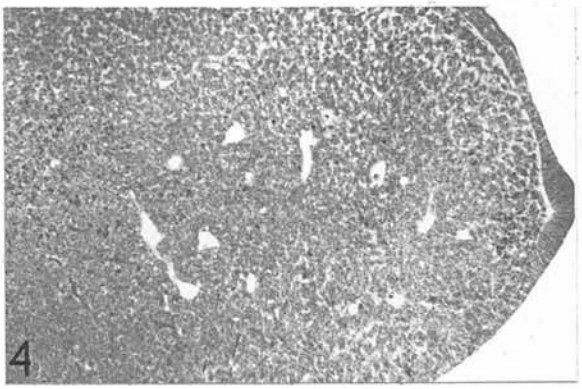

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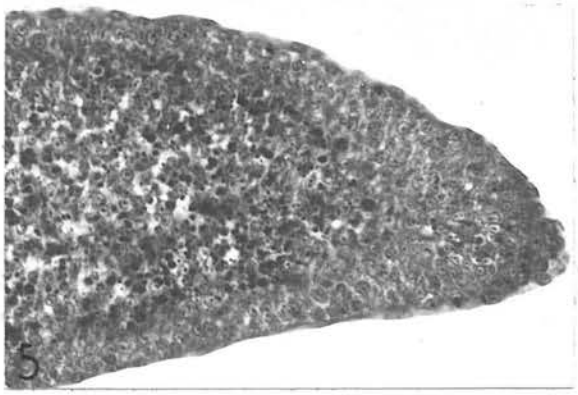

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The 4-day-old embryos, as compared to 3-day-old embryos, seem to be slightly less susceptible to hydrocortisone acetate, and need slightly larger doses. A detailed study done on embryos injected with 7.5 mg hydrocortisone per egg, showed the necrotic areas to be located in the apical and/or central limb mesenchyme (plate 32, figs. 4 and 5). In addition macroscopically visible haemorrhages are also easily detected in the histological preparations (plate 32, fig. 1) and are often accompanied by intensive necrosis in the central mesenchyme of limb (plate 32, fig. 2).

The onset of necrosis is marked by the increased pyroninophilia of cell cytoplasm and appearance of a few dark bodies in it (plate 33, figs. 4 and 6). It is followed by the emargination of chromatin, karyorrhexis and fragmentation of the nucleus into small fractions, up to 10 in number. Meanwhile, the cytoplasm gradually becomes pyroninophobic. The process of necrosis is completed by bursting of the cells and release of nuclear and other debris (plate 32, fig. 5; plate 33, figs. 1 and 3). In addition, one can frequently see macrophages of various sizes containing semidigested and undigested cells as well as apparently healthy looking cells (plate 33, figs. 2 and 4). The macrophages also contain a very large number of rounded darkly staining bodies, which probably are the remains of the digested cells. It is not certain whether the macrophages also engulf the debris of necrotic cells.

The haemorrhage is usually filled with tightly packed blood cells (plate 32, fig. 3; plate 33, fig. 5). Some loosely packed cells in various stages of necrosis are also seen, particularly near the junction of healthy tissue with the haemorrhagic tissue (plate 33, fig. 3).

Plate XI

Figure 1. High power light microscope of the central limb area in plate 52 figure 2. The very extensive necrosis is evident. A number of cells are represented only by darkly staining bodies of cellular debris. MT, X 350.

Figure 2. Part of a section of a 4-day-old chick embryo injected with 7.5 mg hydrocortisone and incubated for further 24 hours. This microgram shows macrophages (arrows) containing few to many cells in the process of digestion. MT X 1,000.

Figure 3. Part of a section of an embryo treated in the same way as the embryo in figure 2. Notice the healthy ectoderm covering the haemorrhage, presence of necrotic cells at the junction of haemorrhage with healthy mesenchyme, and the release of debris by necrotic cells (arrows). MT X 1,000.

Figure 4. Micrograph from the same embryo as in figure 3. The picture clearly shows the presence of a darkly staining necrotic centre in a cell with apparently healthy looking nucleus (arrow). Also notice that a healthy looking nucleus is present in a macrophage (arrow). MT X 1,350.

Figure 5. Micrograph from the same embryo as in figure 3. Part of the haemorrhagic area showing the slightly-clotted blood cells that usually occupy such areas. MT X 1,000.

Figure 6. This micrograph is also from the same embryo as in figure 3. It shows cells that have necrotic centres in their cytoplasm (arrows). MT X 1,400.

Figures 7 and 8. Right and left views of the trunk of an embryo treated in the same way as the embryo in figure 2. A concentration of the blood limbs in two areas points to the micromelia of right hind limb in figure 7. X 7.5.

Plate 33

Figure 1. High power light micrograph of the central limb area in plate 32 figure 2. The very extensive necrosis is evident. A number of cells are represented only by darkly staining bodies of cellular debris. M7, X 350.

Figure 2. Part of a section of a 4-day-old chick embryo injected with 7.5 mg hydrocortisone and incubated for further 24 hours. This micrograph shows macrophages (arrows) containing few to many cells in the process of digestion. M6 X 1,050.

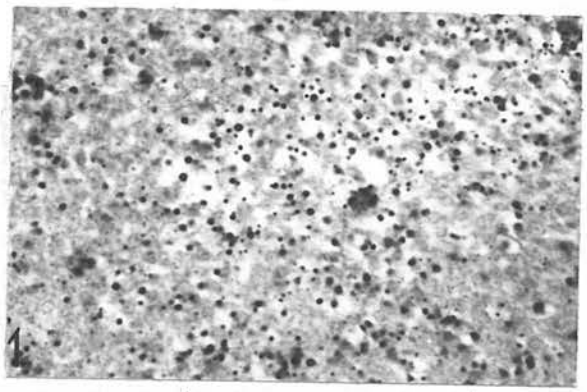
Figure 3. Part of a section of an embryo treated in the same way as the embryo in figure 2. Notice the healthy ectodern covering the haemorrhage, presence of necrotic cells at the junction of haemorrhage with healthy mesenchyme, and the release of debris by necrotic cells (arrows). M7 X 1,000.

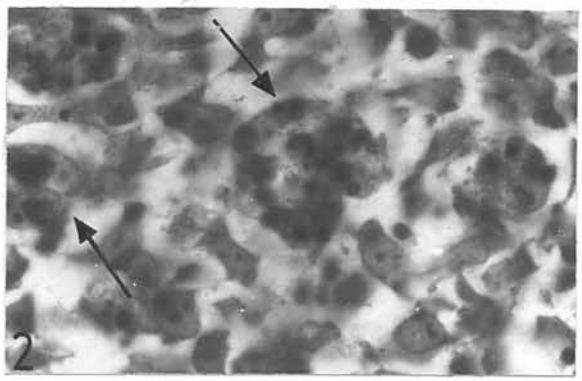
Figure 4. Micrograph from the same embryo as in figure 3. The picture clearly shows the presence of a darkly staining necrotic centre in a cell with apparently healthy looking nucleus (arrow). Also notice that a healthy looking nucleus is present in a macrophage (arrow). M7 X 1,300.

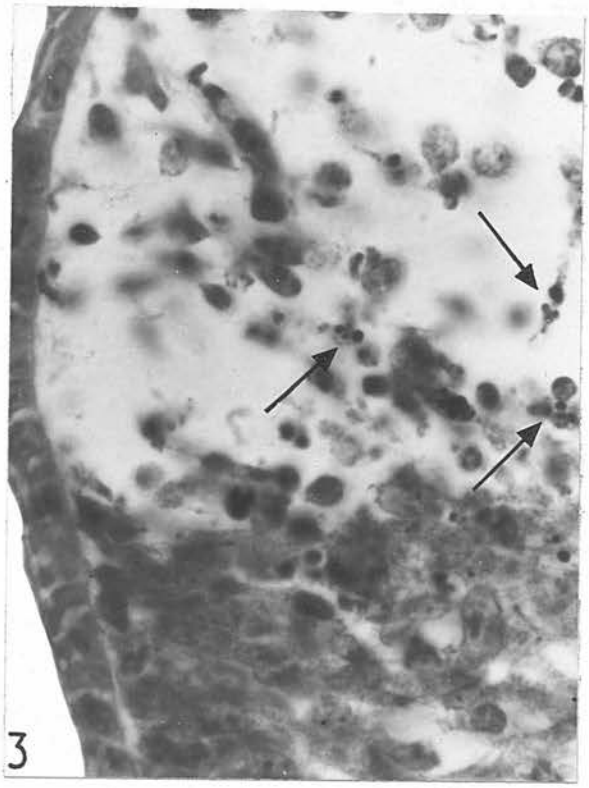
Figure 5. Micrograph from the same embryo as in figure 3. Part of the haemorrhagic area showing the tightly-packed blood cells that usually occupy such areas. M7 X 1,000.

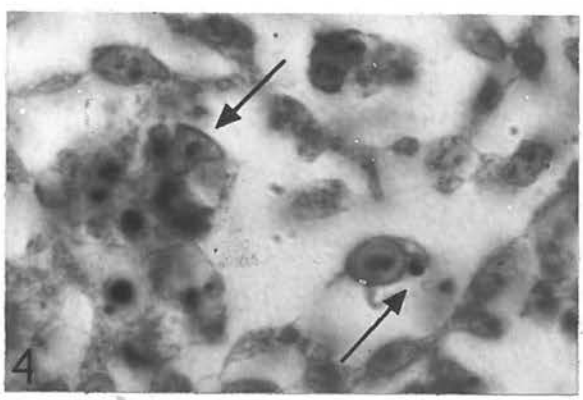
Figure 6. This micrograph is also from the same embryo as in figure 3. It shows cells that have necrotic centres in their cytoplasm (arrows). M7 X 1,400.

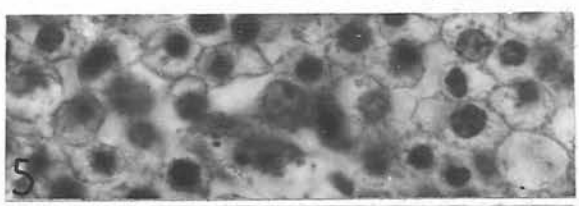
Figures 7 and 8. Right and left views of the trunk of an embryo treated in the same way as the embryo in figure 2. A comparison of the hind limbs in two cases points to the micromelia of right hind limb in figure 7. X 7.5.

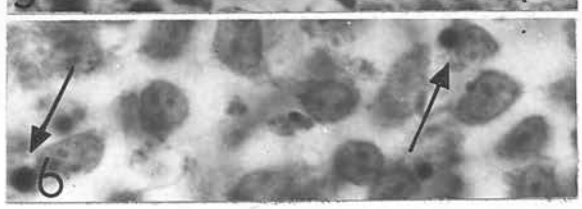


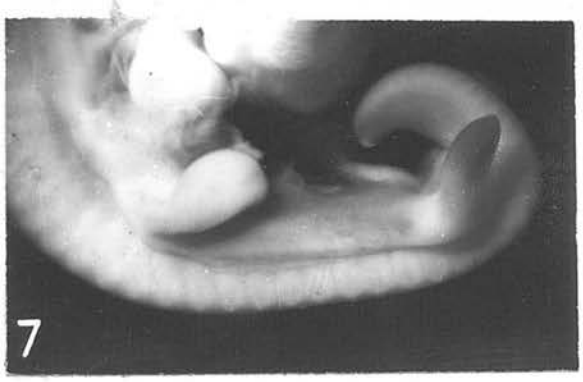


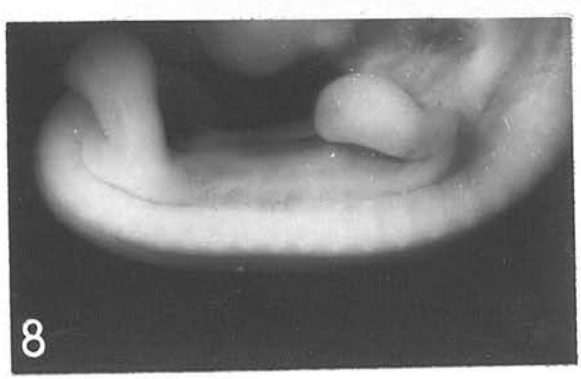












D I S C U S S I O N

1. DIFFERENTIATION OF THE CELLULAR PHASE OF CARTILAGE

I. Mesenchyme cells

In the mesenchyme cells as in a variety of other undifferentiated cell types - for example developing liver cells (Howatson and Ham, 1955), blastema cells (Hay, 1958), developing cnidoblast and gonial cells (Fawcett, 1959), developing aorta cells (Karrer, 1960), developing notochord cells (Jurand, 1962), neurala stage cells (Perry and Waddington, 1966), developing myoblasts (Manasek, 1968) and undifferentiated plant cells like those of the root cap (Whaley, Kephart and Mollenhauer, 1964) - the endoplasmic reticulum system is sparce and unorganised, consisting mainly of smooth vesicles that are sometimes slightly elongated. This type of endoplasmic reticulum is considered to be characteristic of cells which do not synthesize proteins for extracellular use (Haguenau, 1958; Fawcett, 1959,66; Porter, 1961, 64).

The presence of numerous free-lying polysomes, is further evidence that the cells are undifferentiated and are actively engaged in the synthesis of only endocellular proteins (Godman and Porter, 1960; Porter, 1961, 64; Nørrevang, 1968). The Golgi apparatus of the mesenchymal cells consists of vesicles and a few flattened lamellae. This underdeveloped state of the Golgi apparatus is usually interpreted as indicating that such cells are not involved in the synthesis of extracellular proteins (Hay, 1958; Godman and Porter, 1960). The cytoplasmic ground substance, or hyaloplasm, of mesenchyme cells, particularly the early mesenchyme cells, is almost electron translucent. This property of cytoplasm is common to other embryonic cells and has been related to "an extraction effect resulting from higher water content than that of differentiated cells, and a lower

concentration of constitutive or structural protein" (Godman and Porter, 1960).

The nuclei of the mesenchyme cells described here, as also of those described by Fell (1925) and Godman and Porter(1960), are comparatively large, usually irregular in shape and centrally placed. Such nuclei are also typical of other undifferentiated cells, for example, blastema cells (Hay, 1958), presumptive notochord cells (Jurand, 1962) and cultured amniotic cells (Anderson, 1967).

The presence of nuclear pores is a feature common to all types of cells. The structure and function of the pores are important because the pores seem to constitute a potential avenue for communication between nuclei and cytoplasm. In our work the nuclear pores in the early mesenchyme cells of the chick contain an electron dense material and have a condensed diaphragm or septum across each. In other cells investigated in this study, the diaphragm is nearly always missing and the pores are usually filled with material whose electron density is either similar to or lower than that of the material on either side of the pore. Several other workers have reported the presence of diaphragms in nuclear pores in various tissues (Afzelius, 1955; Watson, 1959; Merriam, 1961; Fawcett, 1966); a central dot- or granule-like structure in the pores, however, is reported in tangential sections of nuclear envelopes from salivary gland cells of insects (Wiener, Spiro and Loewenstein, 1965, and others) and some other organisms (Kessel, 1965). The interpretation of the diaphragm as the membranous edge of the pore, either above or below, when the section has been off the centre (Watson, 1959; Kessel, 1965) is untenable in the light of later researches: in particular Fawcett (1966) has shown the presence of "a thin flange that encircles the pore at the level of the diaphragm, projecting from its limiting membrane into perinuclear cisterna". The present as well as other observations are in agreement with Merriam (1961) who considered the diaphragm as a separate structure distinct from the membranous elements of the

envelope. The significance of the presence of the diaphragm only in certain cases in this study is uncertain, but it might be noted that the diaphragm has been reported mainly from studies on oocytes and undifferentiated cells. On the question of communication through the pores, the results of different experiments conducted to measure the potential across nuclear envelope, by placing microelectrodes in it, are not in agreement. It has, however, been suggested "that in most cells the pores are not open to free diffusion but may be the sites of regulated transfer of materials" between the nucleus and cytoplasm (Fawcett, 1966; Wiener, et al., 1965).

The plasmalemma around the mesenchymal cells of the chick and the mouse, as seen under the electron microscope, is a more or less continuous membrane suggesting complete structural independence of these cells. The discontinuities of the plasmalemma are almost certainly preservation artifacts due to the high water content of such cells. The idea of the artificial nature of the discontinuities is supported by the observation that sometimes the plasmalemma is well preserved at the sites of cell-to-cell attachment while it is missing at the sites where the cell is adjacent to the extracellular phase (plate 20, fig. 1). This observation is in accord with that of Godman and Porter (1960) and supports the view of Fell (1925) that these cells have a structural independence. Therefore, the view held by some histologists (Schafer, 1949) that undifferentiated mesenchyme cells form a syncytium seems untenable.

II. Differentiation of chondrocytes and hypertrophy

Cellular differentiation or the assumption of a specialised function by a cell, is heralded by sorting out of heterogeneous cytoplasmic organelles into recognisable structural groups which are capable of a specific function. These changes mainly concern the membrane system of the cell; but changes in the

cytoplasmic ground substance and nucleus are also discernible.

The formation and development of the endoplasmic reticulum system, with its granular cisternae, is one of the first signs of differentiation of the chondrocytes. The association between the granular endoplasmic reticulum and the synthesis of proteins for extracellular use has been so well established that the presence of granular endoplasmic reticulum is an acceptable criterion for the synthesis of protein by any cell (Godman and Porter, 1960; Porter, 1964; Fawcett, 1966; Kessel, 1968; Ross, 1968). The morphogenesis of the endoplasmic reticulum is generally considered to proceed in the following steps: (a) adlineation and coalesence of small vesicles to form smooth membraned elongated cisternae of the reticulum, (b) attachment of ribosomes onto the cisternae to form granular cisternae, and (c) characteristic arrangement of the cisternae in the cytoplasm and their dilation into the saccular cisternae (Hay, 1958; Godman and Porter, 1960; Jurand, 1962; Jackson, 1964). Silberberg (1968) considered the dilations into saccular cisternae to indicate either an increased production of protein or retarded discharge of protein from the cisternae. In the present study the contents of the endoplasmic reticulum were always amorphous, homogeneous and moderately electron dense. These observations do not support the reported presence of thin, non-periodic fibrillar structures in the endoplasmic reticulum cisternae by other authors (Revel and Hay, 1963; Silberberg, et al., 1965; Anderson, 1967).

The origin of the endoplasmic reticulum, and in particular of the small vesicles, is not very certain. In the present work some smooth profiles are seen attached to the outer nuclear membrane in the mesenchyme cells; continuity between the outer nuclear membrane and the endoplasmic reticulum is also seen in the later stages. These observations support the idea of the origin of endoplasmic reticulum by means of blebbing activity of outer nuclear membrane (Waddington, 1962; Waddington and Perry, 1962; Kessel, 1968; Nørrevang, 1968), and are

consistent with Porter (1961) who considered the continuity between endoplasmic reticulum and nuclear envelope as one of the best criteria for defining endoplasmic reticulum; he also suggested that endoplasmic reticulum and nuclear envelope should be regarded as one system. Haguenau (1958), in a review paper, discussed other possible sources of endoplasmic reticulum: the nucleus, nucleolus, mitochondria, centrosphere and plasmalemma (see also Hay, 1958; Karrer, 1960; Lindegren, 1962): Whaley et al. (1964) considered it to arise from the ground cytoplasm.

It is suggested that the continuity frequently observed between the endoplasmic reticulum and the perinuclear envelope in the fully differentiated cells (Hay, 1958; Godman and Porter, 1960; Jurand, 1962), and even in the hypertrophic cells as observed in this study, is not due solely to the fact that the endoplasmic reticulum originates from the nuclear envelope. Porter (1961), in a review paper, has already concluded that transport within the endoplasmic reticulum does occur. It is quite plausible that the continuity serves a useful purpose in allowing the contents of the reticulum to come in contact with the nucleus through the inner nuclear membrane and to exert an influence on the genetic activity at the transcription level. This idea becomes all the more attractive when one considers that the chromatin is distributed adjacent to the inner nuclear membrane; this means that the cytoplasmic inducer or repressor substances, that are needed to activate or inactivate the operon (Jacob and Monod, 1961), are separated from the operon only by a unit membrane, namely the inner nuclear membrane. The present idea of the feed-back by contents of reticular cisternae ties up with the suggestion made by Waddington (1962) that the endoplasmic reticulum may influence the activities of the chromosomes through the nuclear envelope. Two further points may also be indicated here. (1) Though the feed-back mechanism suggested here concerns mainly the influence of newly synthesised proteins in the

reticular cisternae, it does not necessarily exclude other metabolites and non-metabolites from exerting their influence through this mechanism, since they are also known to accumulate in the reticular cisternae after pinocytosis (Palay and Karlin, 1959; Porter, 1961). (2) The nucleo-cytoplasmic interactions and influences exerted through the inner nuclear membrane, as suggested here, are only supplementary to and do not substitute for those exerted through the nuclear pores.

The present study shows that the endoplasmic reticulum is very well developed in chondroblasts and reaches a peak of development in the chondrocytes. These observations are in agreement with those of Godman and Porter (1960). The endoplasmic reticulum persists even in the hypertrophic chondrocytes, as also observed by Silberberg, et al. (1961), Anderson, (1964), Anderson (1967) and Silberberg (1968). Godman and Porter (1960) assumed that the presence of well-developed endoplasmic reticulum even in the hypertrophic chondrocytes indicates not only continued protein synthesis but also segregation and storage of these proteins which may be liberated into the extracellular phase at the time of disintegration of hypertrophied chondrocytes. These authors, however, report a partial collapsing of the saccular cisternae of endoplasmic reticulum in hypertrophic chondrocytes in the case of rat. In our observations on the mouse, on the other hand, it is noted that the endoplasmic reticulum cisternae become dilated in the hypertrophic cells. Similar observations were made by Silberberg (1968) on mouse articular cartilage cells after thyroxin injection and by Palfrey and Davies (1966), Anderson (1967) and Meachim and Roy (1967) on rabbit and human cartilage cells. All these workers considered these changes to be of a degenerative nature.

The arrangement of ribosomes in the differentiating chondrocytes undergoes changes which involve attachment of ribosomal groups, the polysomes, to the endoplasmic reticulum. These changes are characteristically related to the

synthesis of proteins for extracellular use (Porter, 1961; Jamieson and Palade, 1967; Kessel, 1968; Ross, 1968). The continued presence of a large number of free polysomes in the chondrocytes, until they undergo hypertrophy, suggests that the cells remain active in the synthesis of endocellular proteins as well. The disappearance of free-lying polysomes but retention of membrane-bound polysomes in the hypertrophic chondrocytes indicates that these cells are synthesizing proteins only for extracellular use. Furthermore, chondrocytes, like other cells, also show ribosomes attached to outer nuclear membrane (see reviews by Porter, 1961; Nørrevang, 1968). This means that the outer nuclear membrane either serves as an extension of the endoplasmic reticulum (Porter, 1961) or has some special types of ribosomes attached to it (Sager, see in discussion following Swift, 1959).

The organisation and development of the juxtanuclear Golgi apparatus during chondrogenesis follows the development of the endoplasmic reticulum. The Golgi apparatus acquires more lamellae, numerous vesicles and also a number of vacuoles. The frequency and size of the vacuoles increase with progressive differentiation of cartilage and reach a maximum in chondrocytes, where the vacuoles are present throughout the cytoplasm. This course of events was also observed by Hay (1958) and Godman and Porter (1960). The contents of the lamellae and vesicles are moderately electron dense and amorphous. The contents of the vacuoles, on the other hand, are electron translucent in the chondroblasts, but are granular and fibrillar in chondrocytes. The granules and fibrils resemble similar elements of the extracellular phase (Revel and Hay, 1963; Matukas et al., 1967; Silberberg, 1968). The fact that at the time of hypertrophy of the chondrocytes the Golgi apparatus undergoes a marked decrease in size was reported also by Silberberg (1968) among others.

The origin of the Golgi apparatus has been recently reviewed by Beam and Kessel (1968). In the present study vesicular structures similar to the Golgi vesicles have been observed in association with the endoplasmic reticulum. This suggests that the Golgi vesicles originate from the reticulum (Revel and Hay, 1963; Jamieson and Palade, 1967; Kessel, 1968). Such vesicles are also seen flattening and coalescing with each other to form the Golgi lamellae (plate 8, fig. 1). In addition the possibility of derivation of the lamellae from the granular endoplasmic reticulum by detachment of ribosomes is suggested from an observation where the two structures are continuous with each other (plate 8, fig. 2). The formation of the vacuoles and the increase in their size most probably occur by the fusion of vesicles pinched off the Golgi lamellae (Plate 26, fig. 1; see also Godman and Porter, 1960).

The gradual appearance of other cytoplasmic organelles including lipid droplets is another feature of chondrogenesis (Godman and Porter, 1960). The presence of lipids is considered to be a normal and constant feature of chondrocytes (Montagna, 1949; Collins et al., 1965); however, Hay (1958) did not report any lipids in her study of differentiating cartilage. Our observations are more in accord with those of Godman and Porter (1960) made on rat cartilage in that the lipids were not as frequent as in some other animals (Collins et al., 1965). Lipid droplets were almost completely missing from hypertrophic chondrocytes. These facts support the view that the stored lipids are not associated with a degenerative process (Montagna, 1949). Silberberg et al. (1964) and Silberberg (1968), on the other hand, noted that degeneration of cartilage manifests itself by an increase in the number of lipid vacuoles and the appearance of free lipids in the cytoplasm. The significance of the presence of lipids is unknown, though Collins et al. (1965) suggested that the lipids may serve as readily accessible energy sources either for the chondrocytes or for the whole organism. They

also noted that the amount of lipids in chondrocytes is influenced by the following: age of the animal, site of the sample and the position of the chondrocyte in relation to the articular surface or to the perichondrium.

The presence of double membraned pools in differentiating cartilage cells (Godman and Porter, 1960), was not recorded in the present study. The only structures which could be compared with the double membraned pools are the mitochondria in the osmium tetroxide fixed material. The artificial nature of these structures is indicated by their absence from glutaraldehyde- osmium tetroxide fixed material. This observation on osmium tetroxide fixed material also explains the suggestion of other workers that some of the cytosomes are derived from mitochondria (see reviews by Silberberg, 1968; Nørrevang, 1968). However some double membraned cytosomes, different in appearance from the pools (Godman and Porter, 1960; Silberberg, 1968), have been seen in the chondroblasts differentiated in vivo and their frequency in chondroblasts differentiated in tissue culture is much higher.

The frequency and size of the multivesicular bodies increases with differentiation (see also Smith and Farquhar, 1966, in case of pituitary function). They are, however, few in mature chondrocytes and are almost absent in the hypertrophic cells (see also Silberberg, 1968). Their frequency in the chondroblasts differentiated in tissue culture, on the other hand, is much higher. Jackson (1964) also reported numerous vacuolar bodies in chondrocytes differentiated in tissue culture. These are also reported in chondrocytes from a variety of animals (see Silberberg, 1968). Nørrevang (1968) considered this organelle to occur normally as a constant component of animal cells; however, a very high frequency of these organelles is a symptom of unhealthy state of the cell (see Glauert et al. 1969). Even so it is surprising to note that in previous studies on the differentiating

cartilage cells either they have not been reported (Godman and Porter, 1960), or have only been infrequently reported (Hay, 1958; see her micrographs, 8 and 9).

The origin and function of multivesicular bodies is still under discussion. In ours, as in a number of other electron microscopic studies (Silberberg, 1968; Nørrevang, 1968) these organelles appear as closed or opened vesicles. There being no artificial breaks of other membranes in these cells, it is reasonable to assume that the majority of multivesicular bodies are incomplete spherical organelles in vivo (Nørrevang, 1968), and may be in the process of formation (Silberberg, 1968). Our work suggests that these organelles originate from the Golgi apparatus (see page sixty ; and Smith and Farquhar, 1966; Glauert et al., 1969), but their origin from other sources can not be excluded (see Gōshi, 1966; Nørrevang, 1968; Silberberg, 1968; Glauert et al., 1969). No definite physiological function has yet been assigned to these organelles but the present observations on tissue culture cells (see page sixty) support the view that they are related to lysosomes (Nørrevang, 1968; Glauert et al. 1969). Moreover, our observation of a multivesicular body opening to the outside lends support to the view of Silberberg et al. (1964) and Silberberg (1968), who proposed that these bodies might serve as means of intracellular transport.

The glycogen is present in the differentiating epiphyseal cartilage of the mouse from the chondroblast stage onwards. The presence of glycogen in differentiating epiphyseal cartilage of rat (Godman and Porter, 1960) and in various adult cartilages has already been so widely reported that glycogen is considered to be a normal and constant feature of cartilage cells (Montagna, 1949). The present study, in accord with other workers (Harris, 1932; Follis and Berthrong, 1949; Silberberg, 1968), shows that the amount of stored glycogen increases in direct proportion to the age of cartilage cells even when the cells undergo hypertrophy.

This led Harris (1932) to suggest that a possible relationship exists between the distribution of glycogen in cartilage cells and the degree of ossification. Follis and Berthrong (1949) also remarked that "there is a rough inverse relationship to the presence of glycogen and the presence of lime salt deposition in the zone of provisional calcification", and suggested that glycogen is the initial substance in a series of reactions needed for calcification.

In our studies on the differentiating epiphyseal cartilage of the chick no glycogen could be seen at any stage; however, some glycogen is seen in cells of diaphysis where cartilage ossification is proceeding. Hay (1958), working on regenerating limbs of Amblystoma also did not report any glycogen in differentiating cartilage. Anderson (1964) while reporting the presence of glycogen in most cells from the head of a rat femur, also remarked on its absence from some cartilage cells near the articular surface as well as from those in the deeper layers of the zone of proliferation. Matukas et al. (1967), working on the tibial cartilage of chick, also did not report the presence of glycogen. Moreover, Davies et al.(1962) reported the absence of glycogen from the articular cartilage of rabbit. The reason for this discrepancy in the distribution of glycogen in the mouse and the chick in this study, as well as in other cases, is unknown.

The intracytoplasmic filaments, or cytofibrils, were present only in the chondroblasts differentiated in tissue culture. These cytofibrils were unbanded and were of two types: (a) 5 - 8 mμ thick fibrils which were more frequent and were distributed in parallel arranged bundles in the perinuclear cytoplasm, and (b) 12 - 16 mμ thick fibrils which were less frequent and present only in peripherally situated cells of cartilage nodules. The occurence of fibrils in chondrocytes, particularly in perinuclear cytoplasm, has been widely reported; for example, articular cartilages of rabbit (Barnett, Cochrane and Palfrey, 1963;

Collins et al.,1965; Palfrey and Davies, 1966), of man (Meachim and Roy, 1967) and mice (after various treatments, see Silberberg, Hasler and Silberberg, 1965; Silberberg, 1968), and in amphibian limb cartilage (Revel and Hay, 1963). Similar fibrils have also been described in other types of cells and in fibroblasts grown in tissue culture (Goldberg and Green, 1964).

The nature and function of the cytoplasmic fibrils is unknown but they have been considered as part of cytoskeleton (Revel and Hay, 1963), as signs of cellular degeneration and ageing (Barnett et al., 1963; Davies et al., 1962) or as indicating metabolic disturbance (see Silberberg, 1968). Meachim and Roy (1967) concluded that the presence of fibrils in small quantities is not evidence of chondrocyte degeneration but that the accumulation of large quantities is indicative of degenerative change; this comment is in accord with our observations. It is important to point out here that none of the above authors has interpreted these fibrils to be precursors of collagen.

In this study fibres, similar to those of the extracellular phase, were also found apparently in the cortical cytoplasm subjacent to the plasmalemma of the chondroblasts. Similar fibres are reported by Godman and Porter (1960) and others (Wassermann, 1954; Yardley, Heaton, Gaines and Shulman, 1960; Porter, 1960; Chapman, 1961) who considered that they are shed by the cell into the extracellular phase to serve as cores of the definitive matrix fibres. The intracellular localization and ecdysis of these fibres is much disputed (see fibrogenesis, page 117 for details). However, the intracellular collagen fibres of adult type with 54 mμ periodicity have been reported by Meek (1965) in fibroblasts of a gastropod. Glauert et al. (1969) also reported collagen fibres, similar to those of the cartilage matrix, in the vacuoles of articular cells grown in culture medium containing 0.08 M sucrose; they explained them as being due to

phagocytosis. It may be mentioned here that the fibres as well as cytofibrils described above seem to be totally different from the felt-like or elastic material reported in the rabbit ear cartilage (Sheldon and Robinson, 1958).

Cellular differentiation is also accompanied by marked changes in the structure of the plasmalemma and the shape of the cell. In contrast to the early mesenchyme cells, the chondroblasts and chondrocytes usually have a well-defined plasmalemma. It does appear, sometimes, indistinct at certain points, but this is probably due to the tangential sectioning or to the opening out of cytoplasmic vacuoles. The chondrocytes usually show shallow indentations on their surface (Silberberg, 1968), but the maturing chondroblasts have characteristic deep indentations of the cell surface (Tousimis and Follis, 1958; Anderson, 1964). Durning (1958) suggested that "the cell extensions are taking part in the process of enlargement of the cell lacunae, perhaps by inducing solution of matrix components and filling out the solubilized area of matrix". He also remarked that the cell processes seen in the osmium tetroxide-fixed material are due to shrinkage effect and may not correspond with in vivo cell extensions, seen in frozen-dried material. Zelander (1959) and Silberberg et al. (1964), on the other hand, pointed out that an irregular surface has a large area in relation to the cell volume; this allows rapid absorption by these cells. Godman and Porter (1960) interpreted this appearance of chondroblasts to be due to the discharge of material by cytoplasmic vacuoles and to the shedding of fibres. Anderson (1964) suggested that the cytoplasmic extensions are the result of the separation of the cells after mitotic divisions. The extent to which each of these factors is responsible for the characteristic scalloped appearance remains to be elucidated.

The differentiation of chondrocytes also involves an increase of the general electron density of the cells, including that of the ground cytoplasm. This

indicates that the process of differentiation is accompanied by progressive dehydration of cells (Scott and Pease, 1956; Hay, 1958; Godman and Porter, 1960). In the case of the mouse this progressive increase in the general electron density of the cells continues during hypertrophy. Godman and Porter (1960), Palfrey and Davies (1966), Meachim and Roy (1967) and Silberberg (1968) noted similar changes in cartilage cells from various mammals, and regarded them as degenerative. In the case of chick, on the other hand, according to the present work, at the time of hypertrophy the cytoplasmic ground substance becomes almost electron transparent and the process is followed by disintegration of the organeller architecture. Scott and Pease (1956), Hay (1958), Takuma (1960), Davies et al. (1962), Silberberg et al. (1964), and Matukas et al. (1967) made similar observations on hypertrophic and degenerative cells of kitten, Amblystoma, rabbit, mouse and chick. This second type of degeneration is explained as being due to a process of hydration following dehydration during chondrogenesis (Scott and Pease, 1956; Revel and Hay, 1963; Silberberg et al., 1964).

The changes in the relative size and intracellular location of the nucleus are other noticeable features of chondrocyte differentiation. The decrease in the relative size of the nucleus is reflected in the change of the nucleo-cytoplasmic ratio from approximately 4 in the mesenchyme to approximately 0.5 in the chondrocytes (see also Godman and Porter, 1960). This is accompanied by a change in the intracellular location of the nucleus from the centre of the cell to an eccentric position within a few hundred Angstrom units of the plasmalemma. This eccentric location was a contributory factor is misleading some earlier workers (Rigal and Little, 1962, among others) into believing that the nucleus of the chondrocytes communicates directly with the extracellular phase. Our observation, after good fixation techniques and high resolving power used in this

study, do not support the opinion that a direct communication exists between the nucleus and outside (see also Silberberg, 1968). During hypertrophy, the shape of the nuclei becomes irregular and the nuclear contents become denser; similar changes were noted by other authors (Scott and Pease, 1956; Palfrey and Davies, 1966; Silberberg, 1968).

The fine structure of nuclear matrix (nuclear sap, karyolymph) varies according to the fixative used (Sabatini et al., 1963; Fawcett, 1966). In some of the present observations on chick chondrocytes, the nuclei fixed in osmium tetroxide alone do not show chromatin while those fixed in glutaraldehyde and postfixed in osmium tetroxide do. This is in accord with the observations of Sabatini et al. (1963) that osmium tetroxide alone is not a reliable fixative for chromatin while fixation in glutaraldehyde results in its adequate preservation. However, in some cases glutaraldehyde-fixation did not distinctly demonstrate any chromatin (plate 4, fig. 3; chick mesenchyme), while in other cases the chromatin was fairly well preserved even by osmium tetroxide alone (plate 22 and 23, figs. 1; chondroblasts from 16- and 18-day-old mouse embryos). The distribution of the chromatin under the nuclear envelope and in small clumps scattered in the nuclear matrix is similar to that reported by other workers in cartilage cells (Rigal and Little, 1962; Silberberg, 1968). The chromatin has also been seen associated with the nucleolus, as noted in other cells (Unuma, Smetana and Busch, 1967). It should be pointed out that like our observations on the chick cartilage, Reval and Hay (1963) also reported diffuse chromatin in younger cells and clumped chromatin pattern in more mature chondrocytes of Amblystoma.

The frequency of mitotic figures decreases with progressive chondrogenesis, but the mitotic figures can be found up to the very last stages studied here, including the chondrocytes laden with glycogen (see also Fell, 1925 and Godman

and Porter, 1960). Montagna (1949), on the other hand, reported amitotic division of glycogen-laden cells.

2. DIFFERENTIATION OF THE EXTRACELLULAR PHASE OF CARTILAGE

I. Development of the extracellular phase

The differentiation of cartilage may essentially be regarded as the process by which mesenchymal cells produce a very extensive extracellular matrix, characteristic of cartilage. The extracellular phase of the prechondrogenic tissue consists of an electron translucent space without any structural matrix. The extent of this space is considerably more in the case of chick than in the case of mouse; Godman and Porter (1960) also reported a comparatively small extracellular space in case the rat. This difference seems to be of no consequence in the later differentiation of cartilage.

During chondrogenesis, the proportion of the extracellular phase, relative to the cellular phase, increases and in fully differentiated cartilage, the extracellular phase usually occupies more space than the cellular phase; however, the proportion depends on the type of cartilage (Revel and Hay, 1963; see also Schafer, 1949). Accompanying this change in proportion of the extracellular phase, is the progressive increase in the electron density of the ground substance and appearance of matrix fibres and granules; similar observations were made by Godman and Porter (1960), in the case of rat chondrogenesis. During chick chondrogenesis, both in vivo and in tissue culture, the deposition of the fibres precedes that of the granules. In the case of mouse, on the other hand, the fibres and the granules seem to be formed at about the same time.

II. Extracellular phase of epiphyseal cartilage

The extracellular phase, both in the chick and the mouse as well as in other species (see Revel and Hay, 1963; Anderson, 1967; Matukas et al., 1968), consists of a ground substance with fibres and granules. In this study it did not contain any lipids or other related substances, as reported by some workers in other species (Montagna, 1949; Ghadially et al., 1965; Anderson, 1967).

The ground substance in this study, as in other studies (Godman and Porter, 1960; Cameron, 1963), is mainly amorphous, slightly electron dense and largely homogeneous, and has some floccules of comparatively higher electron density scattered here and there. Cameron (1963) indicated that much of the ground substance may have been lost in preparing the tissue for sectioning and that in vivo the dense material may have been present throughout the extracellular phase.

The granules of the matrix in this study are usually 20 to 40 mμ in diameter and are found either in association with the fibres or interspersed between them. The frequency and size of these granules varies according to the type of cartilage (Matukas et al., 1967; Matukas and Krikos, 1968; see also Table I). Moreover, the granules are either not reported or reported missing in several studies (Silberberg, et al., 1961; Anderson, 1964; Pihl et al., 1968; see also Table I). Matukas et al. (1967) considered the possibility that the formation of the granules may be due to the clumping of protein-polysaccharide chains, which in vivo extend freely in the interfibrous area of the extracellular phase.

The fibres of the extracellular phase are usually 15 - 20 mμ thick, more or less straight, short and randomly arranged; almost similar results are reported by others (Follis and Tousimis, 1958; Godman and Porter, 1960; Silberberg et al., 1961; Revel and Hay, 1963; Anderson, 1967). The thickness of the fibres is known to range from c.5 mμ to 80 mμ (see Table I), depending mainly on the type

and developmental stage of the cartilage and the distance of the fibres from the chondrocytes (Martin, 1954; Godman and Porter, 1960; Revel and Hay, 1963; Silberberg, 1968). Durning (1958) has suggested that the fibrous appearance of matrix results from fragmentation of the sheets, which he observed in the frozen-dried material. Sheldon and Robinson (1958) also stated that the fibrous network may represent an artifact but they regarded the in vivo matrix to be homogeneously distributed. It seems unlikely that the fibres are an artifact, since similar fibres are seen in other connective tissues (see Jackson, 1964; Porter, 1964), and in cartilage fixed with different fixatives (Luft, 1965; Anderson, 1967; Matukas et al., 1967; present study).

In this study a faint periodicity of 5.5 and 9 mμ has been noted in the fibres of the mouse and the chick respectively. Periodicity ranging from about 8 mμ to the characteristic native collagen periodicity of approximately 64 mμ has been noted by other investigators (see Table I). In general the fibres of articular cartilage have a specific 64 mμ collagen periodicity while the fibres of the epiphyseal cartilage matrix either do not have any periodicity or have a non-specific periodicity (Jackson, 1964). Because the structure of the fibres in embryonic cartilage does not conform with the typical periodicity of native collagen, their chemical nature is in dispute; however, they are generally regarded as collagenous (Godman and Porter, 1960; Cameron, 1963; Matukas et al., 1967; Silberberg, 1968). Martin (1954) studying the sequential appearance of various types of fibres in developing tibia of fowl, was the first to assume that the unbanded fibres of cartilage matrix are not of a collagenous nature. Jackson (1964), from her studies on the residual fraction of protein-polysaccharide from avian long bones, pointed to the possibility of some unbanded fibres being of protein-polysaccharide nature. But Matukas et al. (1967), did not find any

protein-polysaccharide in the fibres after cytochemical investigations. The view that fibres are collagenous is also supported by the observations on other tissues developing in vivo or tissue culture; in these studies similar unbanded or non-specifically banded fibres precede the appearance of characteristic collagen fibres (Porter and Pappas, 1959; Jackson, 1964; Griffin and Harris, 1966; Reith, 1968; see Appendix - Table I).

Two main explanations are usually given to account for the lack of periodicity in fibres of cartilage: (a) the specific banding is missing because the fibres are so thin that there will be insufficient tropocollagen to provide the contrasting arrangement of molecules necessary for the electron microscopic appearance of banding (see Bear, 1952; Ross and Benditt, 1961; however, see also Chapman, 1961), and (b) the banding has been masked by a coating of amorphous soluble substances, presumably protein-polysaccharides, on the collagen fibres (Godman and Porter, 1960; Takuma, 1960; Jackson, 1964; Silberberg, 1968). Furthermore, it should not be overlooked that collagen is a fibrous protein and that the diameter, periodicity and arrangement of collagen fibres depends on the immediate environmental conditions in which fibrogenesis occurs (Wood, 1960; Wood and Keech, 1960; Jackson, 1964; Anseth, 1965). In the light of available evidence, it is safe to conclude that practically all, if not all, the fibres in the extracellular phase are collagenous in nature.

The pericellular capsules, moats or lacunae, of the chondrocytes are generally regarded as specific areas which may have a special type of extracellular matrix (Scott and Pease, 1956; Hay, 1958; Godman and Porter, 1960; Sheldon, 1964; Matukas et al., 1967); and are not considered as artifacts due to shrinkage of cells (Durning, 1958; Silberberg et al., 1961; see also Davies et al., 1962 and Anderson. 1964). As in this study the matrix in the capsules is usually either a normal

extracellular matrix which extends up to the chondrocyte plasmalemma (Sheldon and Robinson, 1958; Takuma, 1960; Anderson, 1964; Palfrey and Davies, 1966), or a largely non-fibrous or finely fibrous matrix (Scott and Pease, 1956; Cameron and Robinson, 1958; Godman and Porter, 1960; Anderson, 1964; Meachim and Roy, 1967; Matukas et al., 1967).

III. Production of the extracellular matrix

Production of the extracellular matrix involves three distinct steps: (1) the intracellular synthesis of the acid mucopolysaccharides, collagenous and non-collagenous proteins or their precursors, (2) the release of these products from the cell, and (3) the appearance of granules and fibres in the extracellular phase. The production of minor components, such as neutral mucopolysaccharides or oligosaccharides for glycoproteins, will not be discussed, but it is reasonable to assume that these carbohydrates are synthesized in essentially the same way as the acid mucopolysaccharides.

(a) The site of intracellular synthesis and accumulation of the products

In this study the autoradiographic observations on cartilage differentiated in tissue culture, using proline -H^3, indicated that collagen of the extracellular phase is synthesized within the cells of cartilage nodule. This is in accord with the evidence available from autoradiographic, tissue culture and biochemical studies on the chondroblasts as well as on other cell types, especially fibroblasts and odontoblasts (Dziewiatkowski, 1958, 1962; Okada, 1960; Revel and Hay, 1963; Godman and Lane, 1964; Fewer et al., 1964; Ross and Benditt, 1965; Neutra and Leblond, 1966a, b; Reith, 1968; Cooper and Prockop, 1968). These authors have shown that the precursors of all the essential constituents of the extracellular phase, including collagen fibres, are synthesized by the connective tissue cells

and not by other cells as believed by some workers (Baitsell, 1915; Nageotte and Guyon, 1934).

Investigations on several different types of cells indicate that the intracellular site of the synthesis of polysaccharides is the Golgi apparatus (Peterson and Leblond, 1964; Neutra and Leblond, 1966a, b; see also review by Beam and Kessel, 1968). The present observations show an acquisition of chondrogen granules, which consist of a number of smaller electron dense granules similar to those of the matrix, by the Golgi vacuoles of the cartilage cells. This lends itself to the interpretation that the Golgi apparatus of these cells contain polysaccharide because Matukas et al. (1967) have shown that the electron dense granules of the matrix are the sites of the main accumulation of protein-polysaccharides. A study of the electron micrographs in other publications (Godman and Porter, 1960; Sheldon and Kimball, 1962; Davies et al., 1962; Revel and Hay, 1963; Matukas et al., 1967; Silberberg, 1968) supports our observations, though the similarity between the subunits of the chondrogen granules and the granules in the matrix is not always mentioned. Moreover, there is autoradiographic and cytochemical evidence for the presence and sulfation of the acid mucopolysaccharides in the Golgi apparatus of chondroblasts (Fewer et al., 1964; Godman and Lane, 1964; Revel, 1964; Matukas et al., 1967).

There is autoradiographic evidence that the collagen protein is synthesized on the ribosomes of the granular endoplasmic reticulum, probably as tropocollagen molecules (Lowther, Green and Chapman, 1961; Revel and Hay, 1963; Kretsinger, Manner, Gould and Rich, 1964; Nichols, 1965; Fernández-Madrid, 1967; see also Ross, 1968). Furthermore, as already indicated, all proteins (collagenous or non-collagenous) needed for extracellular use are supposed to be synthesized by ribosomes attached to the endoplasmic reticulum (Porter, 1964; Karasaki, 1964;

Fawcett, 1966). Jamieson and Palade (1967) showed that such proteins are then transferred to the cisternae of the reticulum. Therefore, the presence of the moderately electron dense material in the cisternae of the endoplasmic reticulum in this study can be interpreted to mean that the cisternal contents are the accumulations of proteins (Revel and Hay, 1963; Nichols, 1965; Ross, 1968).

(b) The intracellular transport and release of the products

The present observations indicate ultrastructural similarity between the vesicles of the endoplasmic reticulum and the Golgi apparatus. Moreover, a direct continuity between the rough elongated cisterna of the endoplasmic reticulum and the smooth lamellae of the Golgi apparatus has also been observed (plate 8, fig. 2). These observations are in accord with the work of Palade and co-workers (Jamieson and Palade, 1967), who presented electron microscopic autoradiographic evidence, in the case of pancreatic tissue, of the active transport of material from the endoplasmic reticulum to the Golgi apparatus through the membrane bounded vesicles. The present observations are also consistent with the assumption that the non-collagenous protein after its synthesis in the endoplasmic reticulum is transported to the Golgi apparatus and here it combines with mucopolysaccharides before being released into the extracellular phase (Godman and Lane, 1964; Neutra and Leblond, 1966a, b; reviews by Ross, 1968 and Beam and Kessel, 1968). Though in this study no Golgi vacuoles could be seen to communicate directly with extracellular phase (see also Silberberg, 1968) merocrine secretion is suggested by the observations in which a Golgi vacuole containing chondrogen granule lies subjacent to the plasmalemma (plate 11, fig. 2). It is quite probable that the process of secretion is rapid or that, due to the scalloped outline of the cell, it is difficult to identify such points of communication once the contact between the extracellular phase and the vacuoles has been established (Silberberg, 1968).

Concerning the intracellular transport and release of tropocollagen molecules the available electron microscopic autoradiographic data, using proline H^3, have been interpreted in three different ways. (1) Revel and Hay (1963), on the basis of qualitative autoradiographic evidence from differentiating cartilage of Amblystoma fore-limb, concluded that the tropocollagen, after its accumulation in endoplasmic reticulum, is transported to the Golgi apparatus before being released into the extracellular phase by merocrine secretion (see also Jamieson and Palade, 1967 for zymogen granules in the pancreatic tissue). They in their study denied any direct passage of the tropocollagen molecule from the ground cytoplasm or from the endoplasmic reticulum. (2) Ross and Benditt (1965) interpreted their quantitative autoradiographic data from wound healing in guinea pig fibroblasts to mean that not all proline-rich protein is secreted through the endoplasmic reticulum-Golgi apparatus route. Ross (1968) suggested a direct secretion of collagenous protein from the endoplasmic reticulum to the extracellular phase. (3) Reith (1968) while interpreting his quantitative autoradiographic results on developing odontoblasts, suggested that there may be more than one route for the release of tropocollagen from the cells and proposed that tropocollagen is secreted directly from the ground cytoplasm to the extracellular phase (see also the review by Cameron, 1963). Cooper and Prockop (1968), working on cartilage, supported his view from their quantitative autoradiographic data. Furthermore, the release of collagen from the ground cytoplasm in a fibrous form (after the dissolution of plasmalemma) has been suggested solely on the basis of some electron microscopic observations (Godman and Porter, 1960; among others ; however, see also reviews by Cameron, 1963 and Jackson, 1964).

In this study, the following observations are probably relevant: the contents of the saccular cisternae of the endoplasmic reticulum always differ from

those of the Golgi vacuoles in their appearance and at times the cisternae communicate with the extracellular phase (plate 7, figs. 1 and 3; plate 24, figs. 2 and 3). The observations may be interpreted to mean that the collagen is secreted directly from the endoplasmic reticulum. Many authors have suggested that there is a direct communication between the endoplasmic reticulum and the extracellular phase (Epstein, 1957; Palay and Karlin, 1959; Karrer, 1960; Porter, 1964; Ross and Benditt, 1965; Nichols, 1965; Ross, 1968) although no electron micrograph showing the communication has been published (however, see Epstein, 1957).

(c) Site of formation of the granules and the fibres

The evidence, from this as well as other electron microscopic studies, indicates clearly that the site of granulogenesis is vacuoles of the Golgi apparatus (Godman and Porter, 1960; Sheldon and Kimball, 1962; Revel and Hay, 1963; Matukas et al., 1967; Silberberg, 1968).

The site of fibrogenesis, on the other hand, has always been a disputed subject and all possible sites - cytoplasm, cell surface and extracellular phase, have been suggested. As detailed discussions and reviews on the subject are already available (Godman and Porter, 1960; Cameron, 1963; Revel and Hay, 1963; Porter, 1964; Jackson, 1964; Ross and Benditt, 1965; Reith, 1968; Ross, 1968), the following account reports only the essentials of the views related to the observations in this study.

Some micrographs, especially of cells differentiated in tissue culture, show certain fine unbanded apparently intracellular fibres which may be continuous with the extracellular fibres. It must be noted that at places where the 'intracellular' fibres are continuous with the extracellular fibres, the plasmalemma appears indistinct or discontinuous. Similar observations on fibres

lying subjacent to the plasmalemma have in the past prompted the idea of intracellular formation of fibres (Wassermann, 1954; Yardley, et al., 1960; Godman and Porter, 1960; Chapman, 1961). These 'intracellular' fibres are supposed to be extruded by the process of excortication (ecdysis or delamination) after the breakdown of the plasmalemma (Godman and Porter, 1960; Chapman, 1961). The author, however, agrees with Jackson (1964) in thinking that other possibilities should be considered before such an explanation is accepted: for example, the spatial relationship concerning the thickness of the sections (70 -90 mμ), the fibre diameter (20 mμ) and the plane of sectioning. Another possibility is the phagocytosis of extracellular fibres or of extracellular materials which may precipitate the tropocollagen intracellularly (Jackson, 1964). The role played by ecdysis in fibrogenesis can not be properly judged, until there is corroborative evidence from cytochemistry and autoradiography.

It has been observed that the extracellular fibres are often adjacent to the plasmalemma at places where there are no 'intracellular' fibres. A similar situation has been described by other investigators (Porter and Pappas, 1959; Karrer, 1960). This suggests that the fibres are formed at, or in close association with, the cell surface. The idea is especially attractive for other reasons as well: (a) at the cell surface the tropocollagen comes into contact with the extracellular environment, which has a different chemical composition and may well be responsible for fibrogenesis (Wood, 1960; Wood and Keech, 1960; see also Jackson, 1964), (b) the extracellular phase may contain additional substances that contribute to the fibres (Jackson, 1956, 64), and (c) the cell surface may act as template (Porter and Pappas, 1959). Reith (1968) proposed a novel hypothesis to explain this observation: the soluble tropocollagen molecule "threads its way through the cell membrane at the expense of time, rather than passing through as a

coiled globular mass, the part of the molecule that is out of the cell would aggregate with other emerging threads because of the extracellular conditions. Or, the emerging thread could precipitate onto a nearby fibre as it increases in thickness ..... The tail part of the emerging thread, however, would still be joined to the cell but not visible in the electron microscope because of its uncoiled nature".

The view that fibrogenesis occurs at some distance away from the cell in the extracellular phase is favoured by Revel and Hay (1963). These authors noticed that proline-H^3, presumably bound in tropocollagen, diffuses readily through the matrix after leaving the cell. From this they proposed that collagen is in the form of soluble tropocollagen when it leaves the cell and precipitates at some distance from the cell. The author takes the view that fibrogenesis "occurs at, or in close association with the cell surface and also at some distance from the cell" (Jackson, 1964).

3. EFFECTS OF HYDROCORTISONE ON THE CHICK EMBRYOS.

Hydrocortisone as well as cortisone are adrenocortical hormones, which are chemically closely related and are quite similar in their effects on embryos (Jurand, 1968); it is suggested that cortisone becomes active only after conversion into hydrocortisone (Cope, 1964).

The present observations indicate that 7.5 mg per egg (LD 50) of hydrocortisone injected into the extra-embryonic coelom on the 4th day of incubation causes a slight retardation of growth and development of limbs, after further incubation for 24 hours. It also causes haemorrhages of limbs and micromelia in some of the surviving embryos.

Landauer (1949), after the injection of adrenocortical extract into the yolk sac of 0- or 5-day-old chick embryos, found retardation of the growth but no

malformations: though he found that similar treatment with insulin leads to malformation. Sames and Leathem (1951) also found that desoxycorticosterone acetate and cortisone acetate injections into the yolk sac of 6-day-old chick embryos caused retardation of body weight increase without inducing abnormalities. Karnofsky et al. (1951), on applying 1 mg per egg of cortisone to the chorioallantoic membrane of the 4-day-old chick embryos, also found a slight growth retarding effect after a further incubation of 24 hours. Other workers have since confirmed the growth retarding effect of adrenocortical hormones on the chick embryos (Moscona and Karnofsky, 1960), chick bone rudiments grown in vitro (Reynolds, 1966) and other organisms, like Drosophila (Smith, Koenig and Lucchesi, 1968). Jurand (1968) working with the foetal mice, however, does not find any retardation of growth after hydrocortisone injections to the pregnant mice.

Karnofsky et al. (1951) and Moscona and Karnofsky (1960) report that comparatively large doses (up to 2 mg/egg) of cortisone in 4- and 8-day-old chick embryos cause various developmental abnormalities involving eyes, feathers, and skeletal tissues. These workers examined the embryos mainly on 18th day of incubation and did not observe any early effects; for example on 5-day-old chick embryos. From our observations, on the other hand, it is clear that large doses of hydrocortisone considerably affected the embryos even after 24 hours of treatment. The malformations in the form of haemorrhage and micromelia as seen in this study are very similar to those noted by Jurand (1968) in the study on mouse embryos after a comparable treatment.

In this study, as in the study on mouse embryos (Jurand, 1968), the mesoderm of the limb-buds, particularly in its distal portion (including the area subjacent to the apical ectodermal ridge), is much more sensitive to hydrocortisone than is the epidermis. In the case of thalidomide injections in the chick embryos, on the other hand, it is the central and ventral mesoderm (away from the apical

ectodermal ridge) which is reported to be more sensitive (Jurand, 1966). Jurand (1966, 68) suggested that the primary sites of action are the endothelial cells of the main blood vessels; this causes necrosis and disruption of endothelial lining which leads to disturbances of the local blood supply. This author considered the necrotic areas in the limb-bud mesoderm to be a secondary effect. Our observations, on the other hand, may be interpreted to mean that the necrotic changes in the mesoderm may be a primary effect, because the necrosis, whether subjacent to the apical ectodermal ridge or in the centre of limb-bud mesoderm, is not preceded by dilation and disruption of the blood supply. This idea is supported by the observation that only necrosis, unaccompanied by haemorrhage, is seen in limb-buds of the chick embryos treated with smaller doses of hydrocortisone. These observations may explain the mechanism of some of the malformations noticed by Moscona and Karnofsky (1960) after cortisone treatment of chick embryos. For example, the absence of distal phalanges rather than the proximal parts of the limb skeleton may well be due to the greater sensitivity of the distal mesoderm to such treatment. Furthermore, most of the malformations reported by Moscona and Karnofsky (1960) are primarily related to mesodermal tissues rather than to ectodermal tissues.

After the realization of the fact that death of cells during development is a normal phenomenon of morphogenesis, the mechanism of cell death has been the subject of recent research (Glücksmann, 1951; Saunders, Gasseling and Saunders, 1962; Zwilling, 1964; Saunders, 1966; Saunders and Fallon, 1967). In this study hydrocortisone injections induced two different types of cell death: (1) phagocytosis, in which apparently healthy cells are engulfed by macrophages and are slowly digested, as evidenced by the fact that the macrophages contain cells in different stages of digestion, and (b) necrosis, associated with pycnosis and fragmentation

of the cell nucleus which is preceded by the appearance of extracellular necrotic centres. These observations are in accord with those of Saunders and Fallon (1967) on cellular death in normal development of chick limb-buds. These authors reported that in cases where cell death proceedes at a slow rate no morbid cells prior to the time of their ingestion by macrophages could be identified, either under the electron microscope or using various histochemical methods. At the time when the cell death occurs at an intense rate, the necrotic eells, including those with pycnotic nuclei, are seen outside the macrophages. These authors explained the latter observation by suggesting that cells are dying at such a rate that the cells undergo necrotic changes even before they are ingested. It is beyond reasonable doubt that after hydrocortisone treatment the mesodermal cells of limb-buds are dying at a greater rate and phagocytosis alone can not cope with it; therefore, phagocytosis and necrosis occur simultaneously.

The mechanism by which hydrocortisone causes cellular death still remains to be examined. This is all the more important since it is widely known that hydrocortisone stabilizes lysosomal membranes, thus preventing the lysosomes from releasing hydrolytic enzymes (deDuve et al., 1962; Jacobson, 1964; Jurand, 1968). This action protects the cells against damage induced by various other factors like irradiation or chemical agents (Weissmann and Dingle, 1961; Weissmann and Thomas, 1963) and is responsible for the widespread use of hydrocortisone in medicine to alleviate cellular injury (see Jurand, 1968). However, it is also well established that hydrocortisone primarily acts by controlling the quantity of specific enzymes, most probably by stimulation of the DNA-dependent-synthesis of RNA (Sekeris and Lang, 1964; Kenney et al., 1965 among others). It means that the two effects of hydrocortisone - the protection against cellular injury and the embryotoxic and other toxic effects on cells, can be explained by assuming that

in one case the affected enzymes are constitutive, while in the other case the enzymes are deaminating and proteolytic (Jacobson, 1964; Jurand, 1968).

APPENDIX I

Table I

The range in diameter and periodicity of fibres and size of granules of the extracellular phase of cartilage

Author(s)	Animal	Tissue	Fibre		Granule size (mμ)	Remarks
			Diameter (mμ)	Periodicity (mμ)		
Randall et al. (1952)	Chick emb.	femur	30 - 50	17 in few	-	Squash preparations
	elephant, man and chick	joint surfaces	30 - 50; coarser	18 - 20; 64		
	calf	nasal septum				
Martin (1954)	Chick emb.	epiphysis articular	40 - 40+	x;17;40-40+	-	Some squash preparations; banding increases with age.
	Chick	"	+	x in few; 67 - 69	-	In addition in the early postnatal life some fibres with immature banding are present in the neighbourhood of cells.
		perichondrium	+	27 - 55	-	
Scott and Pease (1956)	Kitten	epiphysis	10	x	-	
Robinson and Cameron (1956)	Human	epiphysis	10 - 20	20 in few	10 - 50	collagen fibre group up to 125 mμ thick.
" " " (1957)	"	"	5 - 25	x	-	
		articular	25 - 50	+	-	
Cameron and Robinson (1958)	"	epiphysis	10 - 25	x	50	
		articular	25 - 50	+	50	
Hay (1958)	Amblystoma	Humerus	10	x	-	

Author(s)	Animal	Tissue	Fibre		Granule size (mμ)	Remarks
			Diameter (mμ)	Periodicity (mμ)		
Tousimis and Follis(1958)	Rat	epiphysis	6 - 20	x	-	
Follis and Tousimis(1958)	"	"	18 - 22	10-13.5; 18-20	-	homogenised and dried cartilage
Zelander(1959)	Mouse, rat guinea pig	articular	8.5; 55	x; 58	-	
Godman and Porter(1960)	Rat young emb.	epiphysis	7 - 12	x	+	
	Rat emb.	"	10 - 18	8; 21	10 - 14	
	Rat	"	15 - 50	+	+	
Takuma (1960)	Mouse	epiphysis	20 - 40	22 in a few	-	
Knese and Knoop(1961)	Rat	epiphysis	20 - 30		-	
Silberberg et al.(1961)	Mouse	articular	10-13, and up to 30;	15 - 30;	-	These fibres are present up to 6 months of age.
			20-40, and up to 80.	60 - 70	-	Such fibres seen from 1 week old mouse onwards; the number of fibres showing subbanding increases with age.
Davies et al.(1962)	Rabbit	articular	8 - 60	10 - 65	-	The diameter and periodicity depend on the zone of cartilage.
Cameron (1963)	Rat	epiphysis	10 - 20	8 - 10	-	
Revel and Hay(1963)	Amblystoma	humerus	2 - 40	x - +	20 - 50	fibres near the cell up to 10 mμ thick and unbanded.
Jackson(1964)	-	articular	60 - 60+	64	-	review paper; banding in non-articular cartilages unspecific if present.
		non-articular	20 - 40	x - +	-	
Luft (1965)	Frog	xiphoid	10 - 18	8 in few	10 - 14	

Author(s)	Animal	Tissue	Fibre		Granule size (mμ)	Remarks
			Diameter (mμ)	Periodicity (mμ)		
Bonucci (1967)	Rat and guinea pig	calcifying epiphysis	7.5 - 25	x;10;21	+	
Anderson (1967)	Mouse	induced cartilage	5 - 10; 25	x; 60	35 X 4-20	unbanded fibres few.
Matukas et al.(1967)	Chick emb.	articular	50	64	x	granules few in intermediate zone.
		intermediate zone	50;20	64; x	+	
		epiphysis	20	21 in few	20 - 40	
		hypertrophic	20	21 in few	20 - 70	
Smith et al.(1967)	Ox	articular	10 - 25; 25 - 90	x;55	-	thin fibres in pericellular capsule only; some of the coarse fibres do not show banding.

The range in diameter and periodicity of collagen fibres during development of non-cartilegenous tissues

Porter and Pappas (1959)	Chick	skin	25 - 120	x - 64	as collagen matures diameter increases and banding appears.
Griffin and Harris(1966)	Human	fibroblast	10;20; 50-80	x;20;64	"
Reith(1968)	Rat	teeth	16 - 50	x - 64	"

+ present

x reported missing

- granules not reported.

APPENDIX II

THE USE OF METHYL GREEN-PYRONIN IN COMBINATION WITH RNASE FOR DETECTION OF RNA IN LIMB-BUDS.

I. Method and observations

The presence of both, RNA and DNA can be cytochemically detected by the use of methyl green-pyronin staining; in this method DNA stains green and RNA red (Brachet, 1953; Pearse, 1960). It must be pointed out, however, that the intensity of staining and probably even the specificity of these stains seem susceptible to various factors; for example, to the fixative used (Kurnick, 1950, 52, 55), dehydration of the material in alcohol solutions (Brachet, 1953), or the state of the staining solution which depends on the number of slides already stained with it.

Fixation with 2.5% glutaraldehyde, rather than tricholoroacetic acid-lanthanum acetate (TCA-LA), short dehydration in alcohol, and the use of fresh staining solution are specially desirable for good results. In sections prepared by this method, the nucleus is seen in varying shades of green with a slight red hue, which is due to the RNA content of the nucleus (Kaufmann, McDonald and Gay, 1948). In some cases the chromatin is seen as one or two additional dark green bodies in the nucleus and in other cases, especially in the red blood cells, it forms a dark green layer just under the nuclear envelope; the mitotic chromosomes and the chromatin bodies in necrotic cells (the degeneration granules of Saunders et al., 1962, who found them to be Feulgen positive) stain very dull dark green. The nucleolus varies in staining from intensely red to light pink. The cytoplasm also shows various shades of red, but in cells undergoing mitosis or necrosis, it is usually intense red.

The TCA-LA fixation alters the final results very considerably. The nucleus appears hyaline or light green rather than green, though its light red hue persists. The small round chromatin bodies of the necrotic cells and the chromosomes of the cells undergoing mitosis stain intensely red to purple, as compared to the dark green colouration after the glutaraldehyde fixation. Almost all the red stain of the cytoplasm is distributed in a reticulate manner, as contrasted to the homogeneously spread red in glutaraldehyde-fixed preparation. Moreover, the variation in the intensity of red in the cytoplasm of various limb-bud tissues, which can be seen in the glutaraldehyde-fixed preparations, is less noticeable. It may also be mentioned here that the matrix of cartilage, like the mitotic figures, stains intensely red.

The specificity of pyronin for RNA, in glutaraldehyde-fixed tissues, was tested by the treatment of sections with ribonuclease. After such treatment, as compared to the control sections treated with distilled water, stainability with the methyl green remains unaffected while that of the cytoplasm with the pyronin is reduced to various degrees (plate 34, figs. 2 and 4). In most cells the cytoplasm is unstained, but in the case of some apical ectodermal ridge cells, single red blood corpuscles and cells undergoing mitosis, the cytoplasm still stains in various shades of red. The pyroninophilia of the nucleolus and the restricted stainability of the nucleus with the pyronin, however, remains almost unaffected. This apparently differential reduction in pyroninophilia of various cell organelles after ribonuclease treatment is probably due to the formation of RNA-protein complexes, so that the RNA is no more open to the action of RNAse (however, see Pearse, 1960). The other reasons may be that the incubation time with ribonuclease was too short or that the cells contain some slightly pyronin-positive but non-RNA material (Brachet, 1953). In any case even the best estimates of RNA

distribution by this technique are only semi-quantitative (Brachet, 1953).

The relative amount of RNA in various tissues was estimated only by visual observations of the intensity of the pyronin stain. The observations indicate that in the limb-buds the maximum amount of RNA is present in the apical ectodermal ridge cells, whose cytoplasm stains very intensely with pyronin (plate 34, figs. 1 and 3). The next in order are the remaining ectodermal cells, followed by the subectodermal mesoderm. In the central mesoderm the prechondrogenic cells contain less than the premyogenic cells. However, as the chondrogenesis proceedes the amount of RNA in the chondroblasts increases.

II. Discussion

As far as the author is aware it is the first time that glutaraldehyde-fixed material has been used in cytochemical investigations with methyl green-pyronin staining and RNAse digestion. Good fixation of nucleic acids achieved by glutaraldehyde fixation is no surprise since Sabatini et al. (1963) have already recommended the use of this fixative for good preservation of chromatin; this study demonstrates that the material is also well preserved for cytochemical investigations with RNAse (see also Jurand, 1965).

The results of study after fixation of material in TCA-LA are also very informative. In our studies it is seen that fixation with TCA-LA for 4 hours at 18 - 20°C, affects the basophilia in general, and the affinity of chromatin for the methyl green in particular. The chromatin now becomes pyroninophilic (see also Kurnick, 1950, 55). Kurnick (1950, 55) made the following assumption to explain the change of affinity for the dye by chromatin DNA: the depolymerized nucleic acids (as after treatment with acids) have a stronger affinity for pyronin while the highly polymerized nucleic acids, as the chromatin DNA, have preferential affinity for methyl green (see also, Pearse, 1960). This apparently explains the

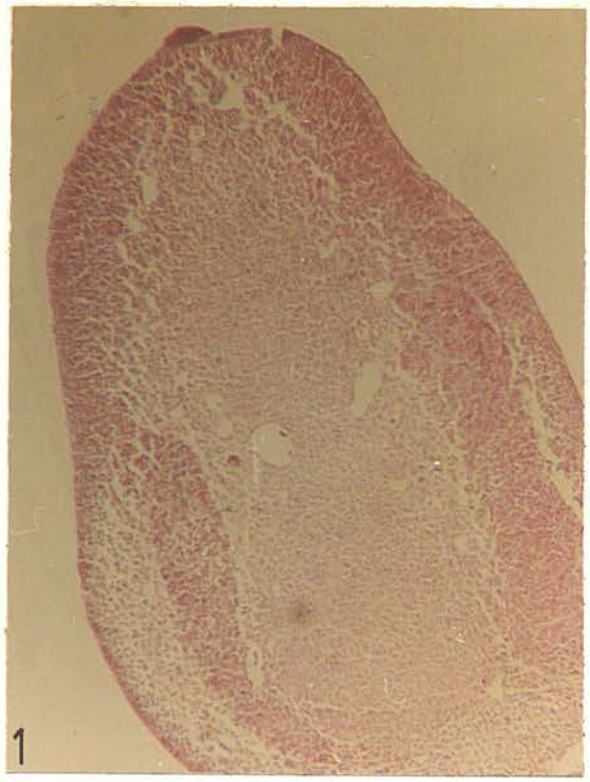
Plate 34

Figure 1. Hind limb-bud of 5-day-old chick embryo showing the relative distribution of RNA, as judged by pyroninophilia. In descending order it is distributed as follows: apical ectodermal ridge, ectoderm, subectodermal mesoderm, myogenic mesoderm and chondrogenic mesoderm. M6, X 110.

Figure 2. Hind limb-bud of 5-day-old chick embryo showing the effect of RNAse digestion on methyl green-pyronin staining. Compare it to figure 1. M8, X 110.

Figure 3. A high power light micrograph of a limb-bud similar to that in figure 1. Note the red cytoplasm and nucleolus, and green nucleus. M6, X 1,100.

Figure 4. A high power light micrograph of a limb-bud similar to that in figure 2. Notice the absence of pyronin stain from the cell cytoplasm, except in some apical ectodermal ridge cells, and its continued presence in the nucleoli. M8, X 1,100.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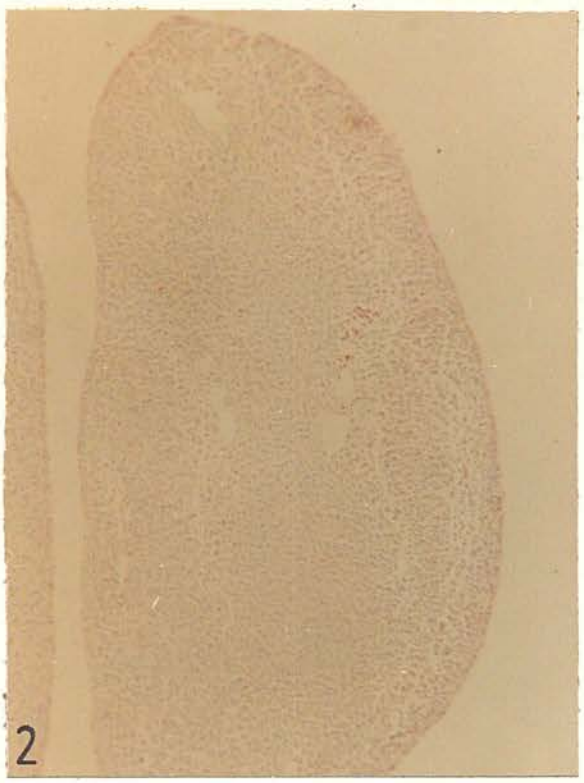
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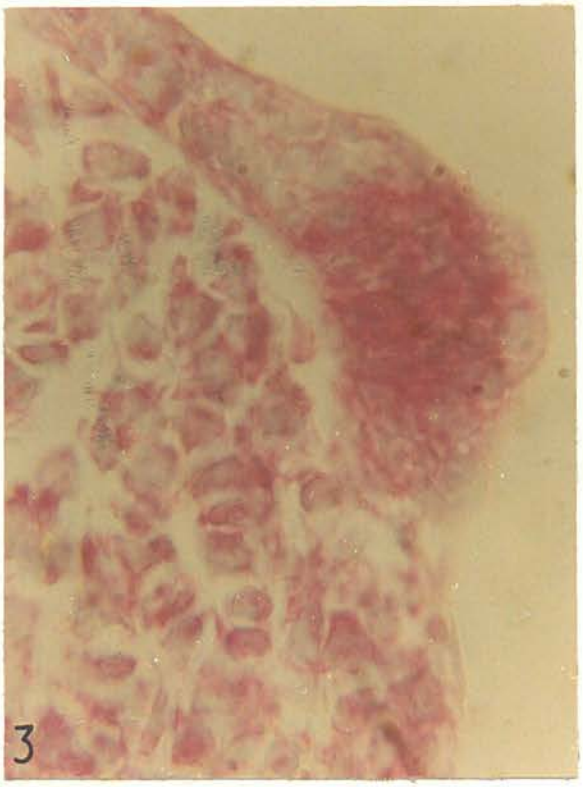
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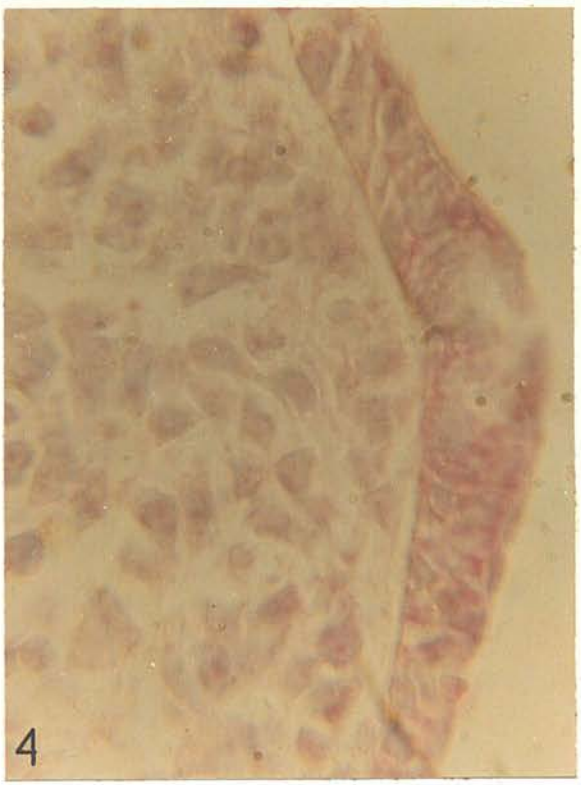
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present findings, except for one observation : after TCA-LA fixation the cartilage matrix, like the chromatin, stains intensely red. This is important since cartilage matrix is one of the very few substances (lignin is another, see Kurnick, 1955), except DNA, that stains vivid green after methyl green-pyronin staining (Kurnick, 1952); this means that the staining behaviour of the cartilage matrix, which is due to acid mucopolysaccharides, is similar to that of chromatin DNA. The explanation offered by Kurnick (1950, 55), therefore, remains open to question till the behaviour of acid mucopolysaccharides is also satisfactorily explained.

The present observations, as well as those of Kurnick (1952), on the cartilage matrix apparently do not support the assumption of Vercauteren (1950). This author suggested that affinity of DNA for methyl green molecule depends on the following stereochemical factor: DNA molecule has negatively charged phosphate residues at a distance corresponding to the distance between the two positively charged sites, that can possibly be present in the methyl green molecule. The loss of affinity of DNA for methyl green is due to the breaking, by acids or other agents, of certain weak hydrogen bonds that are essential to hold the phosphate residues in position. The possibility of the polyanionic mucopolysaccharides of cartilage matrix having stereochemical factors similar to those of DNA and the alteration of these factors by acid fixatives in the same way is not yet clear, but seems unlikely.

The present observations clearly indicate that pyronin is not specific for RNA, and suggest that digestion with RNAse should always be carried out to test the presence of RNA. Our RNAse treatment of sections also supports Pearse's (1960) opinion that the enzyme is specific for RNA and does not affect the nuclear

stains as suggested by Stowell and Zorzoli (1947) and others. The results of distribution of RNA in early chick limb-buds, as described here, are similar to those of Hinrichsen (1956) on mouse limb-buds after gallocynin-chrom alum stain.

APPENDIX III

Published papers:

1. Goel, S.C. and Jurand, A. 1968. Electron microscopic observations on the basal lamina of chick limb buds after trypsin and EDTA treatment. J. Cell Sci. 3, 373-80.

2. Goel, S.C. 1969. Electron microscopic studies on developing cartilage 1. The membrane system related to the synthesis and secretion of extracellular materials. J. Embryol. exp. Morph. 22, (in press).

J. Cell Sci. **3**, 373–380 (1968)
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ELECTRON MICROSCOPIC OBSERVATIONS ON THE BASAL LAMINA OF CHICK LIMB BUDS AFTER TRYPSIN AND EDTA TREATMENT

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SUMMARY

Chick hind-limb buds were treated first with calcium- and magnesium-free balanced salt solution and then with either trypsin or EDTA. Treatment with these chemicals, and the subsequent separation of the ectodermal covering from the mesoderm, produces two different results as regards the basal lamina. In the case of trypsin, the lamina stays with the ectoderm but shows signs of probable tryptic digestion. After EDTA treatment the lamina stays with the mesoderm and becomes very loose and swollen. This loosening allows the structure of the otherwise compact lamina to be resolved so that banded collagen fibrils are seen participating in its substance. The above observations are discussed in relation to the mode of action of the two chemicals. The physiological role and the origin of the basal lamina, as well as the possible implications of the present findings to the question of limb development and morphogenesis, are also discussed. A note has also been made regarding the various terms used to describe the epitheliomesenchymal junction layers.

INTRODUCTION

In the last 20 years tissue culture has become a routine technique in cell biology investigations. To obtain a suspension of cells, disaggregation is effected by treating the embryonic material with various chemicals. Among the most common of these chemicals are trypsin and EDTA (ethylenediaminetetra-acetate disodium salt, or versene). The mode of action of these two chemicals is different, because while the former acts as a proteolytic enzyme, the latter acts through its capacity for complexing calcium ions. In all cases of chemically induced cell disaggregation the affected site is the cell surface or the intercellular cement.

The basal lamina, which is a ubiquitous extracellular layer situated between various epithelia and underlying tissues, and which has recently been the subject of numerous investigations because of its suggested role in limb development (Balinsky, 1956, 1957; Bell, Kaighn & Fessenden, 1959; Bell, Gasseling, Saunders & Zwilling, 1962), is also one of the structures exposed to the chemicals during treatment for cell disaggregation. It was therefore considered desirable to observe the effects of these chemicals on the lamina at the electron-microscopic level.

In contemporary scientific publications the term 'basement' or 'basal membrane' is applied to various, not always strictly comparable, structures. In classical histology it is considered to be a PAS-positive microscopic zone present at the dermo-epidermal

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junction, as well as in other places, where it separates the tissue spaces from 'non-connective tissues' (Low, 1964; Salpeter & Singer, 1959). In electron microscopy, on the other hand, the term is usually applied to 'a continuous membrane covering the basal surface of the basal epidermal cells' (Jurand, 1965; Ottoson, Sjöstrand, Stenström & Svaetichin, 1953) or similar structures (Kurtz, 1958). This is an electron-dense submicroscopic lamina approximately 300 Å in thickness, separated from the basal cell plasmalemma by an electron-transparent space of approximately the same width. The controversy over the usage of the term 'basement membrane' and the consequent suggestion of new terms, like dermal membrane (Selby, 1955), boundary membrane (Low, 1964), adepidermal or adepithelial membrane (Salpeter & Singer, 1959), and basal lamina (Fawcett, 1966), deserve comment. Competent reviews of the terminology have been published by Selby (1955) and by Salpeter & Singer (1959, 1960). The work of Swift & Saxton (1957) has clarified the situation in human scalp and monkey gingiva; they consider the PAS-positive reticulin layer of approximately 0·3 μ thickness as the classical 'basement membrane', and the 300 Å thick electron-dense layer, the 'basement membrane' of electron microscopists, as the 'electron-opaque layer'. Similar investigations, in fact, need to be done for other tissues and species before the results of Swift & Saxton regarding the ultrastructure and terminology of the PAS-positive zone can be generalized. Moreover, as Fawcett (1966) notes, the basal lamina, unlike the plasmalemma and other intracellular 'membranes', is not a trilaminar structure of the lipoprotein nature. We have therefore used the neutral but apt term 'basal lamina', as suggested by him.

It has been suggested that the lamina is a dense mat of exceedingly fine filaments that are tropocollagenous and run mainly in the plane of the lamina; the filaments are either embedded in an amorphous matrix of similar density, consisting mainly of acid mucopolysaccharides, or are very closely compact, so that the individual fibres cannot be resolved (Fawcett, 1966). The precise function of the basal lamina is still largely uncertain, and it is only recently that attention has been paid to it. Caesar & Edwards (1957) suggested that, due to its dense nature and thickness, it protects the 'cell units and their common interspace from too rapid ion concentration changes', while Ottoson *et al.* (1953) and Balinsky (1956, 1957) believed it to form a diffusion barrier, at least in amphibians.

MATERIALS AND METHODS

Brown Leghorn chick embryos at stage 22 of Hamilton and Hamburger were used. The embryos were dissected, and the hind-limb buds were removed by excision. These buds were incubated at 37 °C in calcium- and magnesium-free balanced salt solution (CMF; Moscona, 1961) for 15 min. This was followed, in the case of trypsin treatment, by further incubation for 7–10 min in 1 % trypsin (crystallized and lyophilized, from Worthington Biochemical corporation, Freehold, New Jersey; minimum activity 150 units/mg, I.U.B. system) dissolved in CMF. In the case of EDTA, CMF treatment was followed by incubation for 30 min in 2·7 mM EDTA (disodium salt from BDH, Analar) dissolved in CMF. In both cases the limb buds were then washed

twice or thrice in Hanks's balanced salt solution, and an attempt was made to separate the ectoderm from the mesoderm. In trypsinized buds the ectoderm was easily separated as a loose jacket by using tungsten needles, leaving clean undamaged mesoderm, which is very vulnerable to mechanical damage due to rough handling. In the case of EDTA, on the other hand, scraping with surgical knives was needed to remove the ectoderm, and although a clean mesoderm could be recovered, an intact ectodermal jacket was never separated.

Excised limb buds, kept in culture medium (Moscona, 1961) for the whole time during which the experimental limb buds were treated with trypsin or EDTA, were used as controls. Limb buds which were treated like experimental ones, but in which no ectodermal–mesodermal separation was attempted, were also examined.

For electron-microscopic examination, tissues were fixed for 45 min in chilled (4 °C) 2% osmium tetroxide solution in veronal-acetate buffer (Jurand, 1965) with 0·1% calcium chloride added. After dehydration with graded alcohols (35, 70, 90%, and three changes of absolute alcohol) the tissues were transferred to the Araldite mixture and stirred gently, using a slow rotary shaker (Jurand & Ireland, 1965) for 2–3 h at about 45 °C; the Araldite was changed twice or thrice during this time. The sections (60–90 mμ thick), cut by glass knives on a Porter–Blum ultramicrotome, were mounted on collodion–carbon coated grids, stained with uranyl nitrate and lead acetate (Saito & Matsunaga, 1966) and viewed in an AEI EM 6 or Philips 75 electron microscope.

OBSERVATIONS

In the untreated control limb buds the basal side of the ectoderm is sharply demarcated from the mesoderm, even under the light microscope. In the electron microscope the contour of the basal ectodermal cells on the basal end is very regular and bounded by a continuous electron-dense basal lamina of somewhat fuzzy appearance and approximately 300 Å in thickness (Fig. 1). In contrast, the plasmalemma of the basal cells is very well defined and approximately 100–150 Å thick. Separating the cell plasmalemma from the basal lamina is a uniformly thick electron-transparent space approximately 250 Å thick, so that the basal lamina follows the ectodermal contour very closely. Although the definition of the lamina varies considerably, it is never as good as in mouse limb buds (A. Jurand, unpublished observation). In the untreated buds, because of the dense nature and compactness of the lamina, no filamentous or granular structures could be resolved in the lamina with any certainty. In Fig. 1, below the lamina there is a layer 0·3–0·5 μ thick—probably the PAS-positive zone. This zone, however, does not appear to be continuous, and can well be appreciated only when the section passes in the plane of the longitudinal axis of the fibrillar elements that constitute it; otherwise the fibres are probably cut transversely, and only dense areas of varying lengths appear below the basal lamina.

It was observed that, after trypsin treatment, the basal lamina always stays with the ectoderm (Figs. 2, 3), except when small stretches are missing, probably because of the lytic action of trypsin. The outer surface of the mesoderm always remains

naked (Fig. 4). It seems that trypsin makes the basal lamina swell, since its thickness after trypsin treatment increases to about 400–600 Å. Moreover, due to loosening of the compactness of the lamina, there is a slight indication of the presence of fibrillar elements in it. The electron-transparent space separating the plasmalemma and lamina is still noticeable in most cases.

In the EDTA experiment, during the examination of unstripped buds it was noted that the electron-transparent space was already missing in places, and the lamina appeared loose. After scraping, as pointed out earlier, though a clean mesoderm was available, an intact ectodermal jacket could not be obtained. The basal lamina always stayed as a very prominent uninterrupted enveloping case on the outer surface of the mesoderm (Figs. 5, 6). The treated lamina is about 2000 Å thick and definitely consists of short, straight fibrils and granular particles about 180 Å in diameter. Moreover, at times, distinct banded collagen fibrils 300 Å thick are seen making connexions with the lamina. Whether any change has occurred in the chemical constitution of the lamina cannot be said.

DISCUSSION

The basal lamina follows the ectoderm very closely, and even after mechanical removal of the ectoderm from the mesoderm it stays with the ectoderm (Kallman, Evans & Wessels, 1967). Moreover, when, due to shrinkage, the ectoderm and mesoderm are sometimes slightly separated, the basal lamina stays with the ectoderm (Bell *et al.* 1962). All this indicates that the association of the lamina with the ectoderm is far more intimate than with the mesoderm.

Trypsin, which is a proteolytic enzyme, has at times also been used to test the presence of proteins in tissues (Pearse, 1960). In view of this, the present observations indicate that the proteins susceptible to the action of trypsin are present below the basal lamina rather than in the ectodermal intercellular spaces or in the electron-transparent space. It supports the contention that the intercellular matrix is mainly of mucopolysaccharide nature (Bell, 1960; Fawcett, 1966). However, the loosening and partial digestion of the lamina suggests that some proteins may well be present in it.

Kallmann *et al.* (1967) observed that, when the ectoderm is separated from the mesoderm in the chick limb (tarsometatarsal region of 10-day-old embryos) after treatment with 3 % trypsin pancreatin solution at 3–6 °C, the basal lamina is missing from the basal side of the ectoderm. These authors, however, did not fix the ectodermal tissue immediately after trypsin pancreatin treatment, but after culturing it for at least a day on Millipore filter papers. Neither did they study the free mesodermal surface to ascertain the possibility of the lamina staying with it. Moreover, their material was at a later embryonic stage, and their method of treatment was somewhat different. A critical comparison with our observations is therefore not possible, but it is probable that either the lamina had been dissolved to a large extent, or that it could not be detected because of culturing on filter paper, as the lamina after such treatment is a comparatively loose structure.

As observed by us, as well as by Bell *et al.* (1962), in the case of EDTA treatment

the basal lamina stays with the mesoderm. In the absence of precise knowledge of the chemistry of the electron-transparent space and of the basal lamina itself, nothing final can be said, but the following seems a reasonable explanation, considering the specific way of acting of EDTA and the specific properties of the basal lamina. It is well known that calcium ions are necessary for the maintenance of adhesion of the cells in embryonic tissues, and their absence in the culture medium tends to cause the cell disaggregation (Curtis, 1966). The EDTA has a strong tendency to complex the free calcium ions, so that, in an already calcium-free medium, EDTA tends to complex with all the calcium ions that are released into the medium from the tissue for the maintenance of ionic concentration equilibrium. It is reasonable to assume that the ectodermal cells are the first to be affected by EDTA, and so become detached singly or in groups, from the basal lamina and from the underlying mesoderm. The basal lamina itself is left intact because, due to its dense nature and thickness, it acts as a barrier against rapid changes in ion concentration and protects the underlying mesodermal cells and their common interspace (Caesar & Edwards, 1957).

J. Mulnard & J. Milaire (personal communication), working on mouse limb buds approximately 10 days old, observed however that after trypsin treatment the lamina stays with the mesoderm, while after EDTA treatment it does not, as judged at the light microscopic level. It is difficult critically to compare these observations with ours because of the differences in the material used and in the level of observation.

In the light of the present findings some comments can be made on the physiological role of the basal lamina. Balinsky (1956, 1957) suggested that the absence of basement membrane under regenerating grafts induces the development of limb, and he considered that direct ectoderm/mesoderm interaction is necessary for limb development in the newt. The observations of Salpeter & Singer (1960) on regenerating limbs of the newt show the absence of the basal lamina in the distal part of the early regenerating bud although, as limb regeneration proceeds, a basal lamina is also developed in later stages in a proximo-distal direction. Zwilling (1955), while forming 'composition' limb buds by taking the ectodermal jacket from one limb bud (after trypsin treatment) and the mesodermal mass from another (after EDTA treatment), might well have had two basal lamina in these buds; but, apparently, this also did not affect the development of limbs in any way. Kallman *et al.* (1967) report that the presence or absence of the lamina, in culture studies, in no way affects the orientation or mitotic activity of the ectodermal cells. All this neither supports nor conclusively refutes the hypothesis that the lamina acts as a diffusion barrier of any importance in development (Balinsky, 1956; Ottoson *et al.* 1953). But the results of the EDTA experiment lend support to the hypothesis that the lamina acts as a barrier to rapid ion exchange (Caesar & Edwards, 1957).

The presence of banded collagen fibrils on the epithelio-mesenchymal junction at an early stage of embryonic development, as revealed here after EDTA treatment, may well contribute to the question of morphogenetic role of collagen (Grobstein & Cohen, 1965). But the study of the pattern of collagen distribution at various developmental stages is necessary before any further comments can be made.

As for the origin of the basal lamina, it is considered to arise from the dense

amorphous intercellular ground substance underlying the epidermal cells, but it is not certain from where this substance arises—the ectoderm, the mesoderm or both (Hay & Revel, 1963; Pierce, Beals, Sri Ram & Midgley, 1964). Kallman & Grobstein (1965) interpret the results of their radioautographic work in tissue cultures of mouse salivary glands as favouring 'the hypothesis that basement membrane involves transfer of soluble tropocollagen from the mesenchyme, where it is synthesized, to the epithelial surface, where it is polymerized'. From these studies, however, it is not possible to draw any definite conclusions as to the origin of the basal lamina itself. The study of arrangement and distribution of collagen fibrils in our electron micrographs of basal lamina after EDTA treatment suggests that the basal lamina has a definite contribution from the mesoderm. However, the extracellular nature and very close association of the basal lamina with the ectoderm suggests that the genetic mechanism influencing the formation of the lamina itself might very well operate from the ectoderm.

The authors wish to express their gratitude to Professor C. H. Waddington, F.R.S., for a discussion of the manuscript and to Dr S. Fitton Jackson of the Strangeways Research Laboratory, Cambridge, for her comments. We also wish to thank Miss A. P. Gray for the editorial help.

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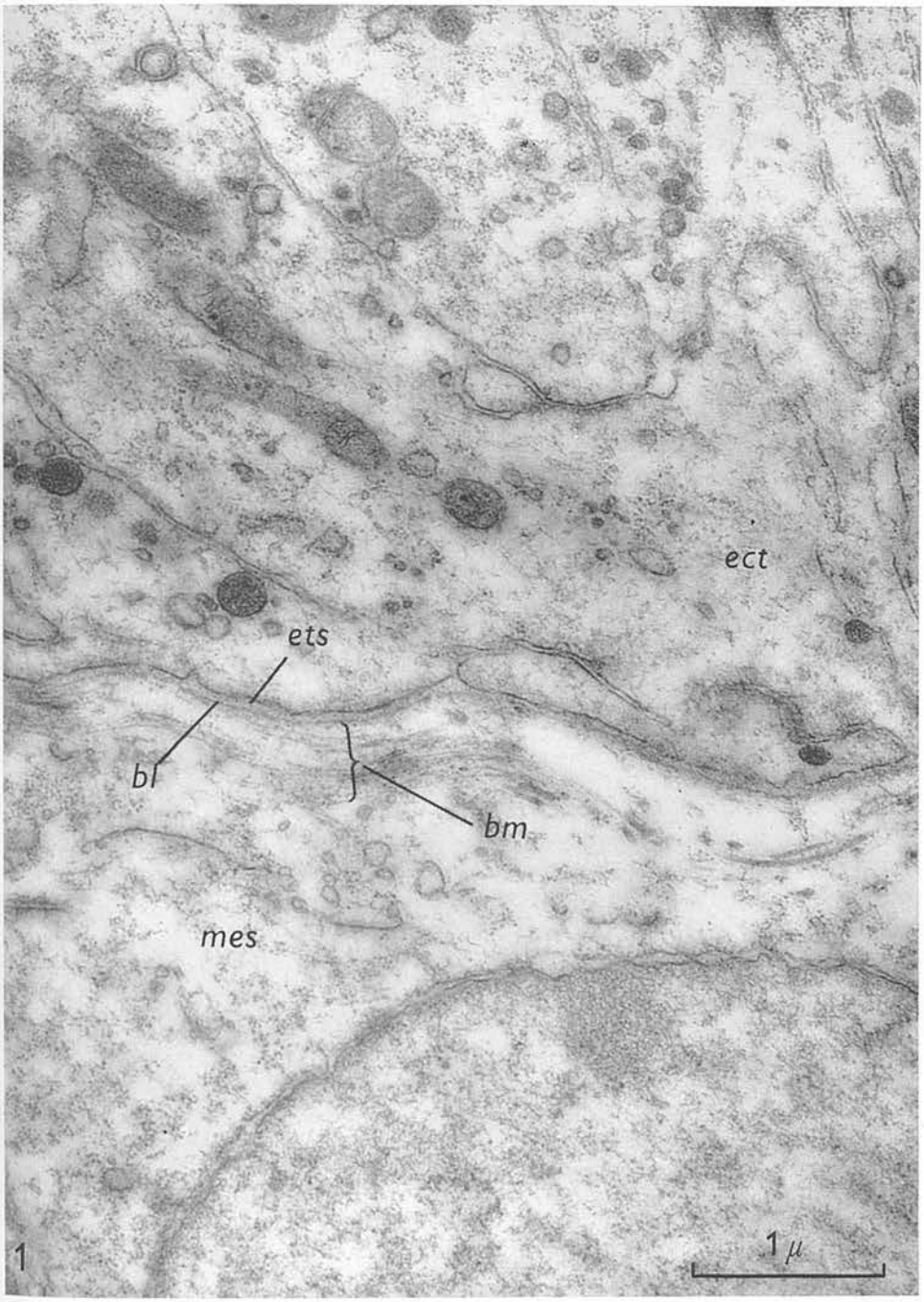
(*Received* 24 *November* 1967)

All sections are of chick limb buds at Hamilton and Hamburger stage 22, fixed in 2 % OsO_4 in veronal-acetate buffer.

Fig. 1. Section through ectoderm (*ect*)–mesoderm (*mes*) junction showing basal lamina (*bl*), electron-transparent space (*ets*) and the classical 'basement membrane' (*bm*). × 26000.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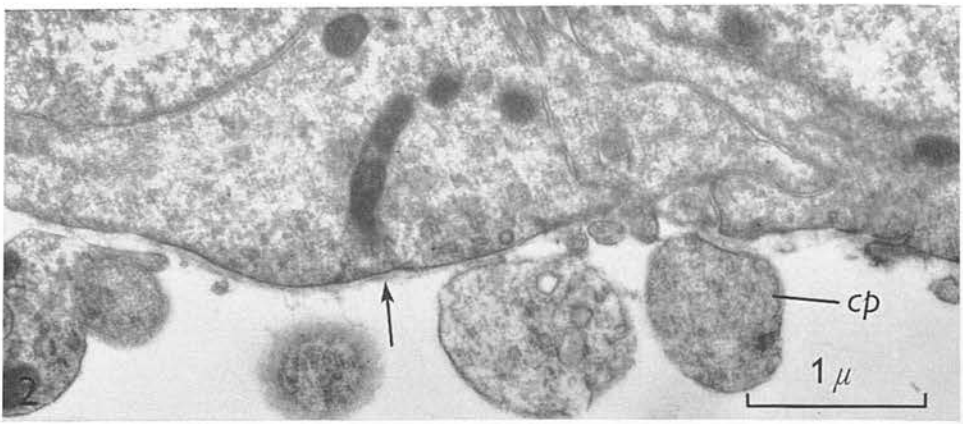
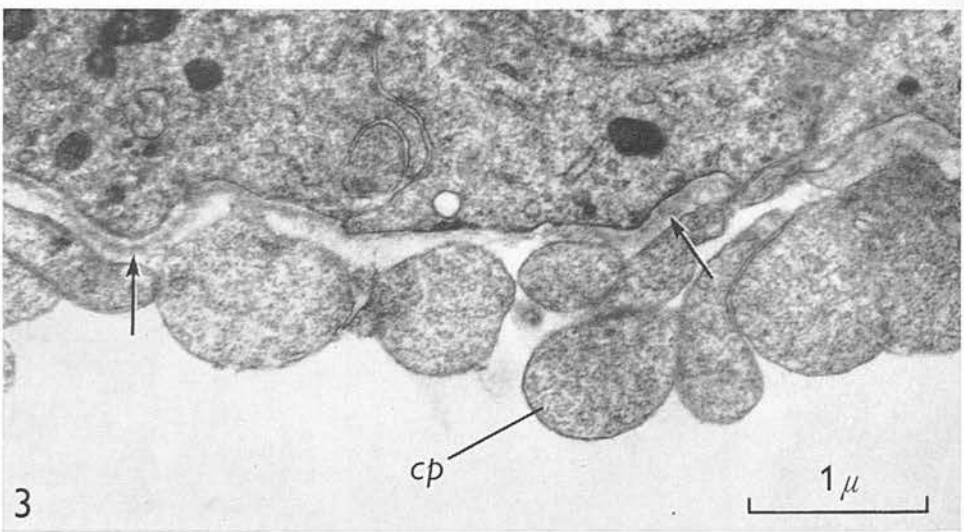
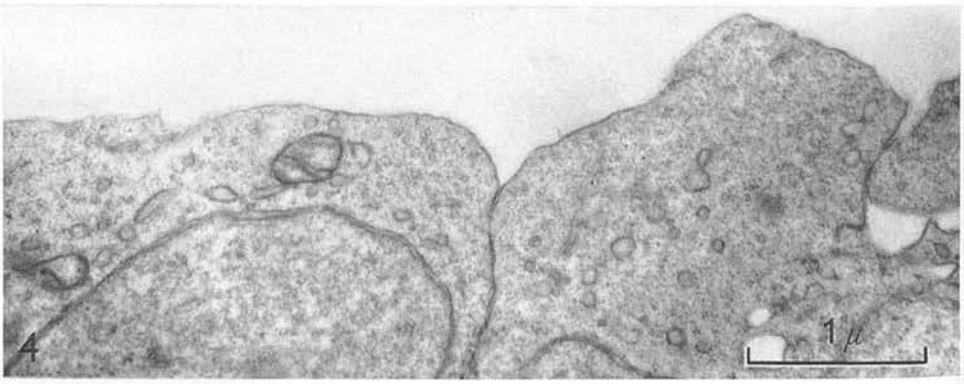
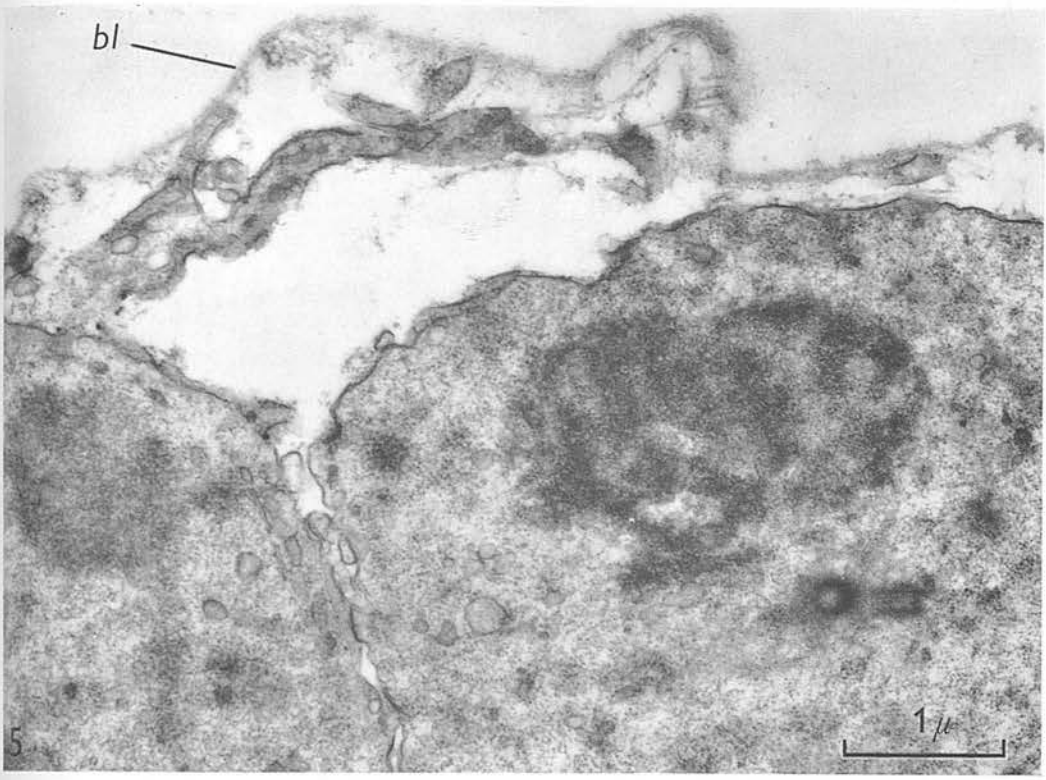






Figs. 2, 3. Sections of the ectoderm separated from the mesoderm after trypsin treatment. A basal lamina (arrows) is clearly seen. Cell protrusions (*cp*) of ectodermal origin are frequently seen. × 23000.

Fig. 4. Section of the mesoderm. The ectoderm has been removed after trypsin treatment. No basal lamina present. × 23000.



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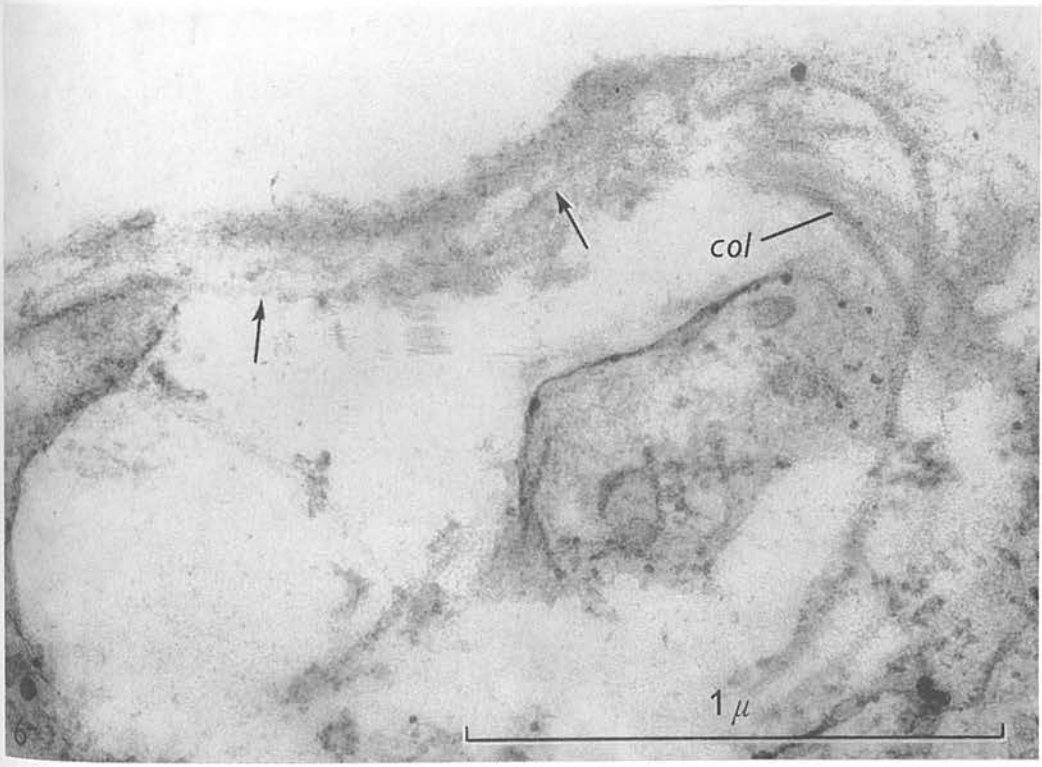

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For legends see next page.

Fig. 5. Section of the mesoderm from which the ectoderm has been removed after EDTA treatment. An uninterrupted swollen basal lamina (*bl*) is present. × 21 000.

Fig. 6. High-power electron micrograph of the mesoderm, as in previous case, to show the structure of the basal lamina (*bl*). Notice the collagen fibrils (*col*) and their merging (arrows) with the basal lamina itself. × 70 000.

LIST OF ABBREVIATIONS ON PLATES

List of abbreviations

c centriole

cg chondrogen granule

ch chondroblast

cr chromatin

cs cytosome

em extracellular membranes

ep extracellular phase

er endoplasmic reticulum

f fibres of the extracellular phase

fn fibrillar region of the nucleolus

G Golgi apparatus

g granules of the extracellular phase

gc ground cytoplasm

gl lamellae of the Golgi apparatus

gv Golgi vacuoles

l lipid droplet

M mitochondria

mv multivesicular body

N nucleus

n nucleolus

ne nuclear envelope

nm nuclear matrix

np nuclear pore

p pinocytotic vesicle

pc perichondrial cell

pl plasmalemma

pn particulate region of the nucleolus

R ribosomes

v Golgi vesicles

List of abbreviations used in explanations to plates only:

M 1 Osmium tetroxide fixation, Araldite embedding and staining with uranyl acetate/nitrate and lead citrate for electron microscopy and with toluidine blue for light microscopy.

M 2 Like M 1 except osmium tetroxide fixation was preceded by fixation in 2.5% glutaraldehyde in sodium phosphate buffer.

M 3 Like M1 except that the fixation in osmium tetroxide was preceded by fixation in 6.5% glutaraldehyde in Millonig's buffer.

M 4 Like M 2 except that the sections were treated with periodic acid and stained only with lead citrate for electron microscopy and were stained with PAS-reagent for light microscopy.

M 5 Like M1 except that the staining was done with uranyl acetate - potassium permanganate.

M 6 Glutaraldehyde fixation, paraffin embedding and methyl green-pyronin staining.

M 7 TCA-LA fixation, paraffin embedding and methyl green - pyronin staining.

M 8 Like M 6 except that, prior to staining, the sections were treated with RNAse.

M 9 Osmium tetroxide fixation, Araldite embedding, digestion with α-amylase and staining with PAS-reagent.

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